Acta physiol. scand. 1959. *46*. 97—106.

From the Department of Clinical Chemistry, University of Lund, Lund, Sweden.

Distribution of C^{14} in Rats after Intravenous Injection of Non-Esterified Palmitic Acid-1-C^{14}.

By

SIGFRID LAURELL.[1]

Received 25 November 1958.

Abstract.

LAURELL, S. Distribution of C^{14} in rats after intravenous injection of non-esterified palmitic acid-1-C^{14}. *Acta physiol. scand.* 1959. *46*. 97—106. — The metabolism of non-esterified fatty acids in the plasma was studied by investigating the distribution of intravenously injected non-esterified palmitic acid-1-C^{14} in rats. Lipids from different organs were studied for activity directly or after separation by means of chromatography on silicic acid columns. The bulk of the activity was found to be taken up by the liver and muscle tissue. A minor fraction was recovered in the fat depots. The results suggested that the fat depots might take up non-esterified fatty acids from plasma without simultaneous esterification. A fair proportion of the palmitic acid was retransported in the plasma glyceride fraction after esterification, probably in the liver.

In recent years several authors have produced more or less indirect evidence in support of the assumption that the major part of non-esterified fatty acids (NEFA[2]) in the plasma is derived from

[1] Present address: Central Hospital of Östersund, Östersund, Sweden.

[2] Now used instead of UFA in order to avoid confusion with Unsaturated Fatty Acids (DOLE 1958).

fat depots. (For a recent review see DOLE 1958.) Experiments carried out by HAVEL and FREDRICKSON (1956) and personal investigations (LAURELL 1957) have shown that the turnover of NEFA in the plasma in dogs and in human beings is large enough to satisfy the bulk of the caloric requirements during fasting.

The present investigation, which is a continuation of previous studies, is concerned with the distribution of palmitic acid-1-C^{14} after intravenous injection in albumin-bound form to (a) glucose fed rats and (b) starving rats.

While the experiments were in progress BRAGDON and GORDON (1958) reported a similar investigation. The present paper not only confirms some of their results but also gives an analysis of the incorporation of the injected palmitic acid in different fat fractions as well as preliminary results of an investigation of the return of the palmitic acid in esterified form into the blood stream. A similar investigation has been carried out by DOLE (1958) but no details of his investigation have as yet been published.

Experimental.

The solution containing palmitic acid was prepared as previously described (LAURELL 1957). Palmitic acid-1-C^{14} of two different specific activities was purchased from Amersham, England. The first batch had a specific activity of 2.81 mc per mM. Of this palmitic acid, 0.73 mg (8 μc) dissolved in 1 ml of 10 per cent human albumin was used for experiments no. 1, 2, 4 and 5. The second batch had a specific activity of 20 mc per mM. Of this palmitic acid, 0.128 mg (10 μc) dissolved in 1 ml of rat serum was used for experiments no. 3, 6, 7 and 8. The exact amount of the solution injected was calculated from the weight of the syringe before and after the injection and from the specific weight of the solution.

White male rats weighing between 150 and 210 g were used for the experiments. During the last 20 hours before the experiments the glucose fed rats were allowed to eat glucose and starch *ad libitum* but nothing else. The starving rats were deprived of food for 48 hours before the experiments. The animals were anesthetised with nembutal (40 mg/kg) injected i.p. The anesthetic administered to the glucose fed rats was dissolved in 10 per cent glucose, while that used for the starving rats was dissolved in saline (vol. injected about 2.5 ml). The palmitic acid solution was injected into an operatively exposed neck vein. Twenty minutes later the animals were bled via a catheter introduced into the aorta. The blood was collected in ice-cooled centrifuge tubes, containing EDTA-Na_2 as anti-coagulant. The liver, 2 g of muscle tissue from the hind legs and all the fat depots except the panniculus adiposus

(isolated according to REED *et al.* 1930) and the plasma were extracted with chloroform-methanol 2 : 1 after homogenisation. The extracts were purified by the method of SPERRY and BRAND (1955) with M/30 KH_2PO_4 instead of water, a procedure involving no loss of non-esterified palmitic acid.

After evaporation of the extracts *in vacuo* the isolated fats were separated into phospholipids and non-phospholipid fats by means of chromatography on silicic acid columns according to BORGSTRÖM (1952 a).

The non-phospholipid fractions were separated into NEFA and neutral fat by means of Amberlite IRA 400 and in some of the experiments also into a cholesterol-ester fraction and a glyceride fraction. After hydrolysis according to SCHMIDT-NIELSEN (1943) the neutral fat fraction and the cholesterol-ester fraction were separated into unsaponifiable fat and fatty acids as described by BORGSTRÖM (1952 b).

The skin with the panniculus adiposus, the inner organs and the rest of the carcass were digested for 16 hours at 100° C with 4 ml of 40 per cent KOH per gram of tissue. After acidification to pH 1 the hydrolysate was extracted twice with an equal volume of petroleum ether. The petroleum ether extracts were afterwards washed with water and 0.0001 N sulphuric acid in 50 per cent ethanol, dried with Na_2SO_4, evaporated and weighed. In Table I the weights of these fats are given as fatty acids without correction for unsaponifiable lipid or esterified fatty acids. The values obtained by this procedure are about 10 per cent too high for the fatty acids from the panniculus adiposus and somewhat less so for other fractions except those from the brain.

In experiments 7 and 8 the distribution of the activity among the plasma fat fractions was studied in glucose-fed rats at various intervals after the injection of 10 μc palmitic acid. 0.1 ml of plasma was obtained from blood drawn from the tip of the tail. A final larger specimen was collected from the aorta. This specimen was used not only for isotope determination but also for quantitative analysis of the fat fractions. In these experiments NEFA and neutral fat were separated by extraction with alkaline ethanol according to BORGSTRÖM (1952 b). The extractions were carried out in glass-stoppered centrifuge tubes.

Analytical methods.

Esterified fatty acids in the non-phospholipid fractions were determined, dissolved in toluene, as an iron complex of hydroxamic acids according to GODDU, LEBLANC and WRIGHT (1955). NEFA was determined titrimetrically after isolation. The phospholipids were determined according to ALLEN (1940) but with a final volume of only 10 ml.

Isotope technique. — The isotope measurements were carried out with a thin mica window GM tube (background about 32 cpm) in the distribution studies, and in a windowless gas-flow GM tube (background about 22 cpm) in the experiments with series of blood samples from one rat. At least 3,000 counts were counted. All samples were plated on

Table I.

	Exp. no. 1 Weight 156 g			Exp. no. 2 Weight 160 g			Exp. no. 3 Weight 175 g		
	Fatty acids mg	cpm per mg	C^{14} % recovered	Fatty acids mg	cpm per mg	C^{14} % recovered	Fatty acids mg	cpm per mg	C^{14} % recovered
Liver NEFA	—	—	0.4	—	—	—	1.1	1,020	0.09
" TG.	39	3,630	19.8	35	5,160	25.3	55	6,090	28.4
" PL.	138	980	19.0	117	1,000	16.3	138	1,280	14.9
" unsap.	—	—	0.069	—	—	0.094	1.0	82	0.007
Muscle NEFA									
" TG.	59	27.7	0.23	32	101	0.45	39	61	0.20
" PL.	17	54	0.13	20	75	0.21	22	99	0.18
Carcass	3,270	47	21.3	6,530	29.3	26.7	4,340	76	28.0
Int. fat NEFA	—	—	—	—	—	—	3.0	226	0.06
" " TG.	2,350	23.1	7.7	3,130	11.2	4.9	4,640	12.4	4.8
" " PL.	49	116	0.8	27	221	0.80	71	120	0.7
Skin	4,460	9.5	6.1	5,970	9.7	8.2	8,580	7.5	5.4
Heart	—	—	—	19.7	385	1.10	—	—	—
Lung	—	—	—	40.5	342	1.95	—	—	—
Spleen	—	—	—	12.2	150	0.26	—	—	—
Digestive tract	—	—	—	323	93	4.3	—	—	—
Kidneys	—	—	—	37.9	246	1.30	—	—	—
Testes	—	—	—	58.0	7.6	0.06	—	—	—
Brain	—	—	—	107	4.2	0.08	—	—	—
Int. organs	722	74	7.6	—	—	(9.1)	1,140	119	11.5
Total recovery	—	—	83.1	—	—	91.9	—	—	94.2

Distribution and recovery of C^{14} 20 min after the injection of palmitic acid-1-C^{14} in glucose fed rats.

C^{14} % recovered = C^{14} recovered as lipids expressed in per cent of the amount administered.
TG. = triglyceride fatty acids, except in exp. 6, including cholesterol esters.
PL. = phospholipid fatty acids.
Unsap. = unsaponifiable lipids.
Int. fat = fat depot lipids except those from panniculus adiposus, which is included in "skin".

lens paper covered aluminium planchets after addition of oleic acid to a final weight of 15 mg per planchet (surface 4.7 cm^2). (ENTENMAN *et al.* 1949.) The reproducibility of the isotope determinations was almost as good as that theoretically possible. However, systematic deviations were noted for fat from organs digested with KOH. Thus the values noted for fat from the carcass and internal organs were about 10 per cent higher, and from the skin about 7 per cent lower than on addition of an equal amount of active palmitic acid to pure oleic acid or to neutral fat from the internal fat depots. This is most likely due to differences in the position of the fat in the lens paper with consequent differences in self-adsorption. This source of error has not been analysed sufficiently to justify the introduction of correction factors but it is not of such an order as to impair the validity of the results obtained.

Results and Discussion.

Broadly speaking, the distribution of C^{14} in the various organs studied was in accord with what BRAGDON and GORDON found (1958) 10 min after the injection of 1 μc palmitic acid. In their investigation, however, only about 1 per cent of the injected dose of palmitic acid was found in the depot fat in glucose fed and fasting rats as against an average of 12.6 per cent and 6.6 per cent in the present investigation. This difference may at least partly be explained by the approximation made by BRAGDON and GORDON (1958) in assuming that the specific activity (expressed as activity per gram wet tissue) of all fat depots was equal to that determined for epididymal or perirenal fat tissue. Thus in one experiment I found the specific activity of perirenal fat to be only 45 per cent of that of pooled internal fat. Furthermore, these authors underestimated the amount of total fat tissue by neglecting its content of water and nonlipid substances, which in my experience comprise about 50 per cent of the fat tissue weight.

The large difference in the recovery of administered isotope from the glucose fed rats (mean 89.4 per cent) and starving rats (mean 54.7 per cent) is in good agreement with the finding of MCCALLA, GATES and GORDON (1957) that the oxidation of palmitic acid-1-C^{14} is much faster in starving rats (about 30 per cent of the injected activity was recovered as expired $C^{14}O_2$ within 20 minutes) than in glucose fed rats (about 3 per cent expired $C^{14}O_2$ within 20 minutes). This implies that the recovered activity in the glucose fed rats was still bound chiefly to the palmitic acid-1-C^{14}, while it is more difficult to say how the situation was in the starving

rats. This may be elucidated by the observation that the incorporation of C^{14} in the unsaponifiable fat fraction of liver fat, which represented mainly steroids, was higher in the fasting rats than in the glucose fed animals. This result is thus the reverse of what is obtained on administration of C^{14} labelled acetic acid, but appears to be in good accord with the modern concept of cholesterol synthesis (LYNEN, HENNING, BUBLITZ, SÖRBO and KRÖPLIN-RUEFF (1958). No significant activity was found in unsaponifiable fat from muscle, or in the cholesterol esters from liver or muscle.

The present investigation provides no information on the extent to which the difference between the distribution pattern of palmitic acid in the starving rats and in the glucose fed rats can be ascribed to differences in the rate of oxidation of palmitic acid in the various organs. However, the activity recovered from the starving animals was on the average about 35 per cent lower than from the glucose fed animals. Still larger differences were found between the triglycerides in the liver, lipids from the heart and from the fat depots. The liver and the heart oxidises fatty acids rapidly and as to the fat depots, it appears reasonable to assume that the liberation of large amounts of NEFA from the fat depots to the blood stream during starvation is accompanied by a decrease in the uptake of NEFA rather than by an increase in the oxidation of fatty acids. In agreement with the findings of BRAGDON and GORDON (1958) it does, however, appear that NEFA is directed to organs requiring energy and that only a minor portion is transported directly to the fat depots.

It is clear from Tables I and II that the small amount of NEFA recoverable from the liver and muscle has a specific activity approximately equal to that of the phospholipids of these organs. It is therefore possible that the occurrence of NEFA in these organs is ascribable to an artefact due to autolysis of phospholipids by the mechanism suggested by FAIRBAIRN (1945). (It should be mentioned that as a rule the liver was homogenised in chloroform-methanol within 2 min after the animals had been bled, the muscle tissue and internal fat depots about half an hour later.) Despite this long period before cessation of the enzymatic processes in the fat depots, the specific activity of the NEFA was much higher than that of the phospholipids and neutral fat from these depots (analysed in exp. no. 3, 5 and 6). Calculations were made of the approximate amount of NEFA in plasma persisting in the fat depots that might have contributed to the radio-

Table II.

Table II.

	Exp. no. 4 Weight 145 g			Exp. no. 5 Weight 150 g			Exp. no. 6 Weight 206 g		
	Fatty acids mg	cpm per mg	C^{14} % recovered	Fatty acids mg	cpm per mg	C^{14} % recovered	Fatty acids mg	cpm per mg	C^{14} % recovered
Liver NEFA	—	—	0.6	2.9	535	0.2	2.2	745	0.14
" TG.	92	3,200	16.7	21	3,710	10.6	30	3,430	8.6
" PL.	137	637	12.0	123	552	3.4	175	1,000	14.9
" unsap.	—	—	—	—	—	0.22	—	—	0.20
Muscle NEFA	—	—	0.008	1.0	89	0.012	2.0	27	0.004
" TG.	26	49	0.18	46	32	0.20	42	81	0.28
" PL.	23	46	0.14	22	97	0.30	30	51	0.12
Carcass	3,460	35.8	17.3	3,030	39.5	16.4	5,050	54	20.0
Int. fat NEFA	—	—	0.10	4.9	242	0.16	6.0	148	0.08
" " TG.	2,510	4.5	1.8	1,470	10.6	2.1	2,140	11.3	2.1
" " PL.	37	62	0.31	29	42	0.17	44	121	0.45
Skin	5,300	5.9	4.2	2,91[illegible]	10.2	4.1	5,180	9.9	4.4
Heart	—	—	—	—	—	—	27.9	111	0.26
Lung	—	—	—	—	—	—	44.3	327	1.2
Spleen	—	—	—	—	—	—	12.8	144	0.15
Digestive tract	—	—	—	—	—	—	272	139	3.2
Kidneys	—	—	—	—	—	—	47.0	251	1.0
Testes	—	—	—	—	—	—	47.1	45	0.18
Brain	—	—	—	—	—	—	144	4.9	0.06
Int. organs	377	107	5.5	673	72.6	6.5	—	—	(6.1)
Total recovery	—	—	58.8	—	—	50.2	—	—	55.3

Distribution and recovery of C^{14} 20 min after the injection of palmitic acid-1-C^{14} in starving rats. See Table I for identification of abbreviations.

Table III.

Exp. no.	NEFA meq/l	NEFA cpm/ml	Neutral fat cpm/ml	Phospholipids cpm/ml
1	—	1,215	326	—
2	0.36	730	1,620	42
3	0.26	1,080	2,400	61
4	0.46	419	224	—
5	0.72	652	219	51
6	0.70	1,040	214	45

Distribution of C^{14} in plasma lipid fractions 20 min after the injection of palmitic acid-1-C^{14} to glucose fed (exp. no. 1—3) and starving (exp. no. 4—6) rats.

activity observed. The calculation was based on the assumption that the amount of blood still in the rat was equally distributed per gram tissue (an assumption that probably gives fictitiously high values for the fat depots), a persistent amount of blood corresponding to 6.7 ml per 100 g minus the amount of blood collected and a hematocrit value of 50 and the activity of NEFA per ml plasma. The results obtained by this procedure showed that the activity of NEFA in the plasma persistent in the fat depots may be equivalent to at most 24 per cent of the activity of NEFA in the internal fat depots in exp. 3, and 2 per cent in exp. 5, and 9 per cent in exp. 6.

The values obtained for the specific activity of NEFA suggest either the occurrence of a pool of esterified fatty acids with a high specific activity, readily autolysed *post mortem* or that the fat depots can take up NEFA from plasma without simultaneous esterification.

The distribution of the activity among the plasma lipid fractions is given in Table III. The results are given in cpm per ml because regular quantitative determinations were only made of NEFA. The most interesting finding was the high activity in the neutral fat in the plasma, especially in the glucose fed rats. In exp. no. 2 the concentration of neutral fat fatty acids in plasma was 1.67 meq/l and the specific activity was calculated as 3,450 cpm per mg. The only isolated fraction with a higher specific activity in that experiment was the neutral fat from the liver (5,157 cpm per mg. Table I).

An attempt to study this phenomenon more closely was made in exp. 7 and 8. The activity in the plasma lipid fractions from two glucose fed rats was followed as a function of time after injection

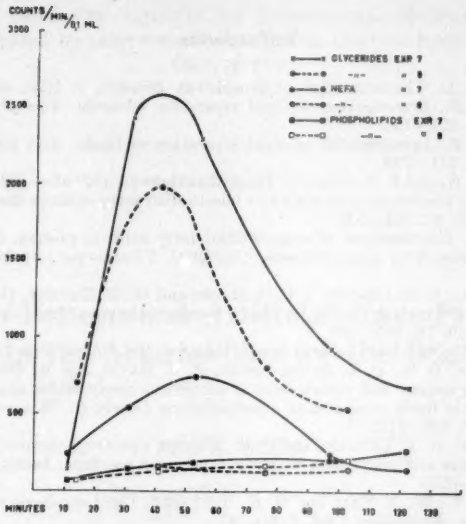


Fig. 1. The activity in the plasma lipid fractions after the injection of palmitic acid-1-C^{14} to glucose fed rats. Exp. 7: glyceride fatty acids: 1.90 meq/l, NEFA: 0.61 meq/l, phospholipid fatty acids 3.43 meq/l.

of palmitic acid-1-C^{14}. The activity in the cholesterol ester fraction was not significant during these experiments. It is clear from Fig. 1 that the curves are substantially similar to those obtained by HARPER, NEAL and HLAVACEK (1953) after administration of labelled acetate to dogs. It therefore appears probable that the part of the plasma NEFA taken up by the liver is directed to the same pools as fatty acids newly synthetised in the liver.

FREDRICKSON *et al.* (1958) have followed the disappearance of injected palmitic acid-1-C^{14} in plasma in dogs for long periods. They found an initial rapid disappearance during the first few minutes and then a somewhat slower phase, and after about 20 min a very slow rate of disappearance. It appears possible that the slowest phase might represent NEFA retransported from plasma triglycerides resynthetised in the liver in analogy with what was found by HAVEL and FREDRICKSON (1956) for the NEFA chylomicron system in dogs.

References.

ALLEN, R. J. L., The estimation of phosphorus. *Biochem. J.* 1940. *34.* 858—865.

BORGSTRÖM, B., Investigation on lipid separation methods. *Acta physiol. scand.* 1952 a. *25.* 101—110.

BORGSTRÖM, B., Investigation on lipid separation methods. *Acta physiol. scand.* 1952 b. *25.* 111—119.

BRAGDON, J. H. and R. S. GORDON, Tissue distribution of C^{14} after the intravenous injection of labeled chylomicrons and unesterified fatty acids in the rat. *J. clin. Invest.* 1958. *37.* 574—578.

DOLE, V. P., The transport of nonesterified fatty acids in plasma. Chemistry of lipids as related to atherosclerosis. *Charles C. Thomas pub. Springfield.* 1958. 189—198.

ENTENMAN, C., S. R. LERNER, I. L. CHAIKOFF and W. G. DAUBEN, Determination of carbon C^{14} in fatty acids by direct mount technique. *Proc. Soc. exp. Biol.* (N. Y.) 1949. *70.* 364—368.

FAIRBAIRN, D., Free fatty acids in animal tissue. *J. biol. Chem.* 1945. *157.* 645—650.

FREDRICKSON, D. S., D. L. MCCOLLESTER, R. J. HAVEL and K. ONO, The early steps in transport and metabolism of exogenous triglycerides and cholesterol. Chemistry of lipids as related to atherosclerosis. *Charles C. Thomas pub. Springfield.* 1958. 205—218.

GODDU, R. F., N. F. LEBLANC and C. M. WRIGHT, Spectrophotometric determination of esters and anhydrides by hydroxamic acid reaction. *Analyt. Chem.* 1955. *37.* 1251—1255.

HARPER, P. V., W. B. NEAL and G. R. HLAVACEK, Lipid synthesis and transport in the dog. *Metabolism* 1953. *2.* 69—80.

HAVEL, R. J. and D. S. FREDRICKSON, The metabolism of chylomicra I. The removal of palmitic acid-1-C^{14} labeled chylomicra from dog plasma. *J. clin. Invest.* 1956. *35.* 1025—1032.

LAURELL, S., Turnover rate of unesterified fatty acids in human plasma. *Acta physiol. scand.* 1957. *41.* 158—167.

LYNEN, F., U. HENNING, C. BUBLITZ, B. SÖRBO and L. KRÖPLIN-RUEFF, Der chemische Mechanismus der Acetessigsäurebildung in der Leber. *Biochem. Z.* 1958. *330.* 269—295.

MCCALLA, C., H. S. GATES and R. S. GORDON, $C^{14}O_2$ excretion after the intravenous administration of albumin-bound palmitate-1-C^{14} to intact rats. *Arch. Biochem.* 1957. *71.* 346—351.

REED, L. L., F. YAMAGUCHI, W. E. ANDERSSON and L. B. MENDEL, Factors influencing the distribution and character of adipose tissue in the rat. *J. biol. Chem.* 1930. *87.* 147—155.

SCHMIDT-NIELSEN, K., Microtitration of fat in quantities of $10.^{-6}$ gram. C. R. Lab. Carlsberg, *Sér. Chim.* 1943. *24.* 233—247.

SPERRY, W. M. and F. C. BRAND, The estimation of total lipides in blood serum. *J. biol. Chem.* 1955. *213.* 69—76.

Acta physiol. scand. 1959. 46. 107—118.

From the University Institute for Experimental Medical Research, Donated by shipowner Anders Jahre, Ullevaal Hospital, Oslo, Norway.

Pulse Pattern and Artificial Wave Pattern in the Arterial Tree of the Dog.

By

PER S. WEHN.

Received 27 November 1958.

Abstract.

WEHN, P. S. Pulse pattern and artificial wave pattern in the arterial tree of the dog. *Acta physiol. scand.* 1959. *46.* 107—118. — Central and peripheral arterial pulse curves in dogs have been recorded synchronously during normal circulation. The peripheral increase in pulse pressure may attain 200 per cent. It is concluded that this increase cannot be due to wave reflection and interference together with a Bernoulli effect and a water hammer effect. The existence of a pulsatory activity of the peripheral muscular arteries must therefore be assumed. Pressure recordings from a large artificial arteriovenous fistula seem to indicate that peripheral wave reflection does not influence the pulse pressure to any greater extent. Central and peripheral pressure curves recorded during extracorporeal circulation clearly demonstrate essential differences between artificial wave motions and the pulse pattern. It is concluded that the physiological pulse cannot be regarded as an ordinary wave motion in the arteries.

Under physiological conditions the pulsatory pressure maximum in the peripheral arteries of the extremities is considerably higher than in the central arteries. This holds also for the pulse pressure

which may be 3—4 times as great in a leg artery as in the aorta. The peripheral increase of maximum pressure and pulse pressure has been described in some detail by FRANK (1905) and confirmed by HÜRTHLE (1934 a), LASZT and MÜLLER (1952) and others.

Similar findings in man have been reported in recent years by SCHNABEL *et al.* (1952) who state that the pulse pressure shows an increase all the way from the aortic valves down to the femoral artery. The mean pressure, however, is practically unchanged. FULLER *et al.* (1952) found that the maximum pressure in the radial artery is higher and the minimum pressure is lower than in the aorta. The pulse pressure therefore, increases considerably, but the mean pressure behaves as the minimum pressure and decreases towards the periphery. KROEKER and WOOD (1955) have recorded central and peripheral pressure curves in man at rest and during exercise, and found that the pulse pressure in the radial artery was 146—165 per cent of the corresponding pressure in the aorta. The mean pressure in the femoral artery was somewhat lower than the corresponding pressure in the aorta and the subclavian artery.

It is easy to confirm these pressure findings by recording the pressure simultaneously in the aorta and in a peripheral artery. A suitable method was used by DAWSON (1906) who put a catheter through the femoral artery into the aorta, and recorded the pressure simultaneously in the two arteries.

Material and Methods.

The dogs used were anaesthesized with Nembutal sodium i. v. 25 mg per kg body-weight, in some cases with additional ether on open mask.

The curves are written by means of a Sanborn 4-channel recorder model 154—100 Bp. An AC-DC preamplifier model 150—1000 was used for the electrocardiograms, and the blood pressure taken by means of Statham transducers P 23 Db in connection with Carrier preamplifiers model 150—1100.

The animals were placed back down with stretched hind limbs so that the aorta, the femoral and the dorsalis pedis arteries lay approximately at the same level. The saphenous artery was then exposed in one leg and a polyethylene catheter introduced through this artery into the aorta. In the contralateral leg the dorsalis pedis artery was exposed and another polyethylene catheter of the same dimensions and length introduced. Some heparine was given to avoid coagulation. Two of the experiments were made during extracorporeal circulation. In these cases the heart-lung machine of DeWall—Lillehei with Sigma

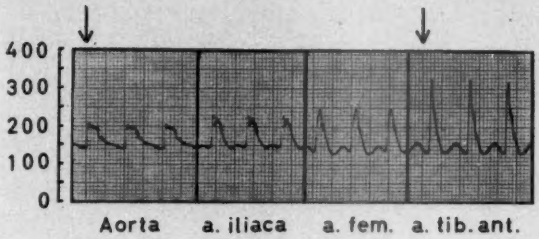


Fig. 1. Dog, 23 kg. Pressure relations in the aorta and the hind limb arteries. Curve segments from the aorta and the tibialis anterior artery were recorded simultaneously. Arrows indicate synchronous points of time.

motors has been used, modified to some extent.[1] The heart was exposed and two polyethylene catheters introduced through the right atrium into the caval veins, leading the blood to the machine. The oxygenated blood from the machine was forced through another polyethylene catheter into the carotid artery. A "positive pressure respirator" was used for the artificial respiration.

Results.

Experiment 1: Dog, 23 kg. The polyethylene catheters were Portex 52 A of length 115 cm. The tip of the one catheter was pushed in 50 cm from the saphenous artery. The other catheter inserted in the dorsalis pedis artery, was advanced some cm, the tip lying in the tibialis anterior artery.

The pressure maximum in the aorta was 205 mm Hg and in the tibialis anterior artery 325 mm Hg. The pulse pressure as will be seen, was about 65 mm Hg in aorta and 195 mm Hg in the tibialis anterior artery. In this case there was no respiratory arrhythmia. The catheter in aorta was now retracted 5 cm at a time during consecutive recording of the pressures. The curves from the tibialis anterior artery remained approximately unchanged. The more central curves, however, show that pulse pressure and maximum pressure increase distinctly as the catheter tip passes the iliac artery and the femoral artery. The minimum pressure, on the other hand, shows a slight decrease.

Experiment 2: Dog, 17 kg. Polyethylene catheters P. E. 90 with length 70 cm were used. That inserted in the left saphenous

[1] Thanks are due to Professor SEMB and Dr. BIRKELAND for permission to do the pressure recordings during their experiments.

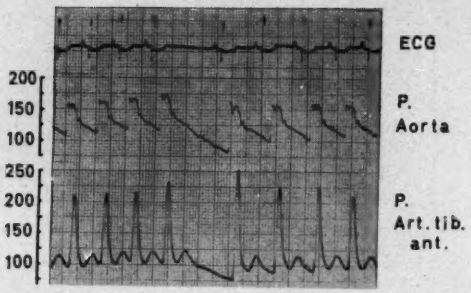


Fig. 2. Dog, 17 kg. ECG, central and peripheral pulse curves recorded synchronously. Definite respiratory arrhythmia, demonstrating that resonance does not influence the pulse pressure.

artery was pushed inwards until the tip lay 45 cm from the arterial incision. The curves given in Fig. 2 show the results. The peripheral pressure curve has an appearance totally different from that of the central curve. The peripheral pulse pressure is about 230 % of the central one. The curves are characterized by a distinct respiratory arrhythmia.

Experiment 3: Dog, 13 kg. In this case the catheter length was 100 cm, type P. E. 190. The catheter in the left saphenous artery was pushed inwards 30 cm to the abdominal aorta. As seen in Fig. 3, the peripheral curve shows a definite pulsus alternans while the central curve shows regular amplitudes. Some seconds later the tibialis anterior curve suddenly became regular, while the experimental conditions remained unchanged. The ECG and the central pulse did not at all change their appearance.

Experiment 4: Dog, 18.5 kg. Extracorporeal circulation as described above. Catheters, P. E. 90 of 70 cm length. The catheter in the saphenous artery was pushed in 35 cm.

Before opening the right atrium the peripheral pulse pressure was up to 2.5 times as great as the central pulse pressure. As soon as the extracorporeal circulation was established, the pressure curves totally changed appearance. During this experiment the circulation through the heart was not totally interrupted, as blood from the sinus coronarius, the small cardiac veins and the bronchial veins had to pass by the ordinary route. The heart was not stopped, and a weak pulse occurred causing spikes in the arterial pressure curves in spite of the extracorporeal circulation. One curve section is shown in Fig. 4. Two types of rhythmical

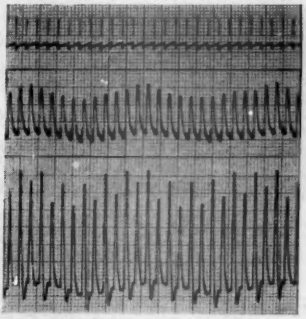


Fig. 3. Dog, 13 kg. ECG with pulse curves recorded synchronously. Pulsus alternans in the tibialis anterior artery, but not in the aorta. Pulse rate 196/min.

pressure variations are seen in the curves. The relatively slow wave shows a frequency of 24/min corresponding to the frequency of the "arterial" Sigma-motor. The more frequent superposed spikes correspond to the heart rate, which was 132/min. The frequency of the respirator was 16/min, however, this frequency is not detectable in the pressure curve. It is clearly seen that the motor-waves have the highest amplitude in the central artery, and the pulse spikes have the highest amplitude in the peripheral artery.

Shortly afterwards the curves given in Fig. 5 were obtained. The frequency of the Sigma-pump was 100/min. The heart-pulse rate, however, does not assert itself in the curves. Still later the curves in Fig. 6 were recorded. Now the pulse spikes have reappeared at a rate of 124/min, while the frequency of the Sigma-pump is 64/min. The pulse spikes show the highest amplitude in the peripheral artery.

Experiment 5: Dog, 12 kg. Extracorporeal circulation as in the previous experiment. However, the dog died on the operating table due to fibrillation of the heart and in spite of heart massage and use of a defibrillator. Nevertheless the operation was completed and extracorporeal circulation started in the dead animal.

The pressure curves given in Fig. 7 were then recorded, showing pressure variations approximately similar to a sinus curve. Pressure maximum and amplitude are higher in the central artery. The flow caused by the Sigma-pump in this case was 1440 ml/min, and the pump rate 110/min.

Experiment 6: Dog, 18 kg. When the dog was 15 months old, an artificial arterio-venous fistula was made on the left femoral

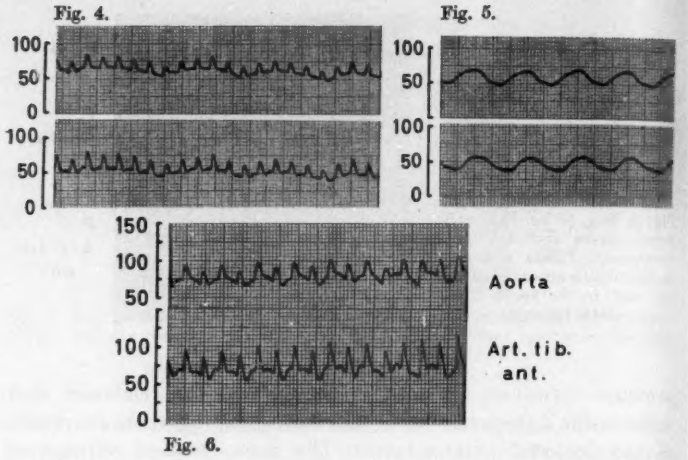


Fig. 4. Dog, 18.5 kg. Extracorporeal circulation. Synchronous pressure curves from the aorta and the tibialis anterior artery. Slow pump waves (24/min) and frequent pulse spikes (130/min).

Fig. 5. Similar curves some time later. Pump frequency 100/min. The pulse spikes have vanished.

Fig. 6. Similar curves still later. Pump waves (64/min) as well as pulse spikes (124/min) are visible.

vessels.[1] The length of the fistula was 15 mm, and it remained patent, the dog being in excellent condition. For the purpose of exact pressure recording the fistula and the right femoral artery were exposed surgically. A polyethylene catheter P. E. 90, of length 70 cm was inserted into the left femoral artery through a small side branch proximally to the fistula. At the same height on the right femoral artery a corresponding catheter was inserted through a similar side branch. In order to record the exact wall pressure (hydraulic pressure) the catheters were placed perpendicular to the arteries, thus avoiding the velocity head pressure. Maximum pressure and pulse pressure was about 10 mm higher in the right artery. Now the left femoral artery was suddenly occluded between the fistula and the site of the pressure recording (indicated by arrow in Fig. 8). The pressure level rose about 20 mm Hg in both arteries. The amplitude, however, remained practically unchanged.

[1] Thanks are due to Professor Ingebrigtsen for permission to utilize this material from his experiments.

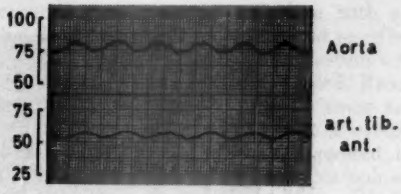


Fig. 7. Dog, 12 kg. Extracorporeal circulation post mortem. Flow 1,440 ml/min. Pump frequency 110/min. The wave amplitude is definitely lower in the peripheral artery.

Discussion.

In healthy dogs the pulse pressure in the tibialis anterior artery ordinarily exceeds the pulse pressure in the aorta by 100—200 %. It has met with great difficulties to give a physical explanation of these pressure conditions. In this connection four possibilities exist: 1. The Bernoulli effect. 2. The water hammer effect. 3. Wave reflection and interference. 4. Pulsatory contraction of the arterial wall.

The Bernoulli equation which reads as follows:

$$v^2/2\,g + p/\gamma + z = k$$

deals with the relation between velocity and pressure when a fluid flows through a tube with varying cross sections.

An approximate calculation based on the pressures in Fig. 1 and a supposed blood velocity of 100 cm per sec in aorta and 10 cm per sec in the tibialis anterior artery shows that the highest possible pressure increase due to the Bernoulli effect will be 3.9 mm Hg. This is a quantity which would hardly be visible in the pressure curve.

The application of the Bernoulli theorem on the arterial tree has been treated by ALOYS MÜLLER (1955) who states that in this case the left side of the equation must be enlarged by three more terms, one due to the frictional pressure loss, another due to pressure loss caused by narrowings, dilatations, branching and arching of the arteries, and a third term due to pressure loss because the flow occurs in spurts. In reality, therefore, a peripheral pressure increase due to the Bernoulli effect is hardly imaginable. At any rate the Bernoulli effect cannot explain the pressure rise in the present experiments because the velocity head pressure is included in the central pressure curve.

The term *water hammer* has been used in rather different ways in medical literature. BAZETT (1924) seems to regard the water hammer effect as a "conversion of kinetic energy into stress", when the streaming fluid meets an obstruction. ALEXANDER (1952) defines the water hammer

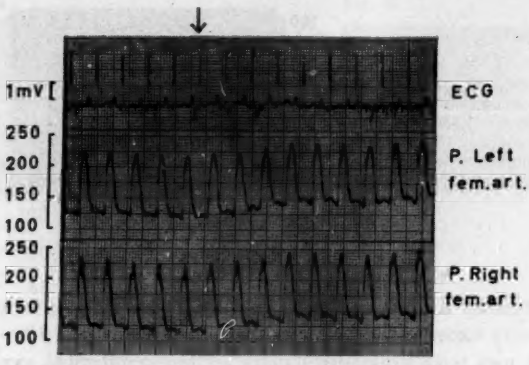


Fig. 8. Dog, 18 kg. Arterio-venous fistula in left thigh. Pressures recorded at a height just centrally to the fistula. Arrow indicates interruption of flow to the fistula.

phenomenon as "the pressure manifestation of a sudden deceleration of the blood ejected by the heart". Another description is given by MALCOLM (1957) who puts the water hammer effect in relation to the cavitation phenomenon when fluid flows in a pipe. GIBSON (1922) and ALOYS MÜLLER (1935) obviously describe identical phenomena, called respectively water hammer and "Wasserschlag", and the following considerations are based on these papers.

A water hammer effect is produced when fluid flows through a rigid tube and the flow is suddenly stopped at the end of the tube by means of a stop-cock. However, if we compare the conditions necessary for a water hammer effect with those existing in the arterial tree, we will find great differences. In the first place the arteries are elastic and not rigid tubes, a fact which will highly reduce a possible water hammer effect. Secondly the pressure rise in the arteries spreads toward the periphery, while a water hammer effect would begin peripherally and spread in central direction. Finally the existence of a pulsatory closing mechanism in the peripheral parts of the arteries or in the capillaries lacks foundation. It must be concluded, therefore, that the conditions for a water hammer effect of this type probably does not exist in the arterial tree.

E. H. WEBER (1851) was the first to apply the general wave theory on the pulse phenomenon. In his opinion the pulse pressure and the maximum pressure have to decrease towards the periphery, and he doubted the finding of any increase (SPENGLER 1843, VOLKMANN 1850, p. 95).

LANDOIS (1872, p. 266) stated as his opinion that the pulse contour depends on local arterial conditions, and that the pulse pattern in one artery is not influenced by wave motions from other arteries.

GRASHEY (1881, p. 174) reckoned with a partial reflection with unchanged phase in the peripheral parts of the arterial tree and re-reflection from the aortic valves. He assumed the existence of reflection with reversed phase when the peripheral vessels were highly dilated. BERNSTEIN (1887) and HOORWEG (1889—1890) were unable to trace any reflection of artificial wave motions in the arterial system of rabbits and dogs. Wave reflection from the bifurcations was suggested by v. FREY (1892, p. 164) but the predominant reflection in his opinion took place in the capillaries where the blood corpuscles practically fill the lumen. Existence of significant peripheral wave reflection was also held by v. KRIES (1892, p. 67) trying to explain in this way a possible peripheral increase of maximum pressure.

In order to explain the peripheral increase of pulse pressure and maximum pressure BRAMWELL and HILL (1923) have suggested a peculiar theory of "breaker" formation in the arteries, due to different velocities of head and tail of the pulse wave. BAZETT (1924) tried to explain the pressure conditions as a result of secondary pulse waves overtaking and interfering with the primary pulse wave.

FRANK (1926) maintains that short waves are generated during opening and closing of the aortic valves. These waves, however, were supposed to be damped and absorbed in the arteries. Only the main pulse wave was considered to be reflected in the smaller arterial branches forming the dicrotism or the arterial "Grundschwingung". WEZLER and BÖGER (1936) assume reflection of the pulse wave with reversed phase at the height of the inguinal ligament. HAMILTON and DOW (1939) reckon with a reflection at the height of the knees, causing a standing wave in the arterial tree, and ALEXANDER (1952) suggests the existence of a standing wave caused by increased resistance and reflection at the height of the femoral artery. HÜRTHLE (1944) gives a series of arguments against wave reflection in the arterial tree, stating that convincing wave motions in central direction have never been demonstrated. Based on mathematical-physical considerations HARDUNG (1952) states that only minute reflection will take place in the arterial tree.

The present material (Fig. 1) demonstrate that the pressure amplitude in the tibialis anterior artery is three times as great as in the aorta. In case of total peripheral reflection, and presupposing that the frictional resistance were zero, the maximal increase of amplitude would be a doubling. A still higher amplitude might be possible in the case of resonance. It appears, however, from Fig. 2 that this phenomenon may be ignored. Due to the respiratory arrhythmia it is directly seen that the amplitudes of one pulse cycle is damped out before the next cycle occur. The existence of a real standing wave, therefore, is highly questionable (VAN DER TWEEL 1957).

The pressure relations shown in Fig. 3 will probably also refute

an explanation based on wave reflection and interference. It is seen that the ECG and the pressure curve from the aorta show regular amplitudes except for the respiratory variations. The pulsus alternans in the tibialis anterior artery therefore, cannot be a passive consequence of the central pulse. The alternans pattern obviously is due to the absence of the dicrotic pressure rise at every second pulse cycle. The phenomenon probably depends on local peripheral characteristics. Similar curves from the carotid and femoral arteries have been published by HÜRTHLE (1934 b).

The curves in Fig. 8 clearly demonstrate that peripheral wave reflection does hardly influence the femoral pulse pressure. The left part of the curves show the pressure conditions while the fistula between the femoral artery and vein was open. In this case wave reflection with inverse phase should be expected. After occlusion of the fistula, we should expect wave reflection with unchanged phase. The amplitudes, however, remained practically unchanged, only the pressure level was raised to any extent. Corresponding pressure relations were found by HÜRTHLE (1944) during pressure recording respectively from the cut and the occluded femoral artery.

Occlusion of an artery will certainly often cause a moderate rise in the pulse pressure. At any rate, however, a 200 per cent increase of the amplitude towards the periphery cannot be due to wave reflection and interference together with a Bernoulli effect and a water hammer effect. Such an increase is only imaginable on the basis of a pulsatory activity of the peripheral muscular arteries (WEHN 1957).

The pressure curves in Fig. 4 clearly demonstrate a reverse relationship between the pulse phenomenon and an artificial wave motion in the arteries. The pump wave shows that maximum pressure and amplitude are higher in the aorta, while the pulse spikes show the highest amplitude in the tibialis anterior artery.

The curve sections in Fig. 5 show no definite pulse spikes. Only the pump waves assert themselves, demonstrating that the maximum pressure is highest in the aorta while the central and peripheral amplitudes are fairly equal.

In Fig. 6 the pulse spikes have reappeared, the amplitudes being highest in the tibialis anterior artery. The amplitude of the pump wave in the latter artery can hardly be evaluated as some dicrotism seems to exist. Pressure curves recorded as soon as physiological circulation was re-established, again showed that

maximum pressure and pulse pressure were by far highest in the peripheral artery. The pressure recordings in Fig. 7 from the dead dog show pressure waves of sinus type behaving exactly as would be expected in passive elastic tubes. Maximum pressure and amplitude decrease towards the periphery. The present material clearly demonstrates that the arterial pulse pattern is characterized by a definite increase of the pulse pressure towards the periphery. Artificial wave motions in the arterial tree on the other hand, never show an increase of the amplitude towards the periphery. This also appears from curves published by LYSLE PETERSON (1954). It is concluded, therefore, that the arterial pulse is not identical with a wave motion in the arteries.

This work has been supported by a grant from Storebrand Insurance Company Ltd., Oslo.

References.

ALEXANDER, R. S., Factors determining the contour of pressure pulses recorded from the aorta. *Fed. proc.* 1952. *11.* 738—749.

BAZETT, H. C., Factors in the causation of differential blood pressure. *Amer. J. Physiol.* 1924. *70.* 550—585.

BERNSTEIN, J., Über die secundären Wellen der Pulscurve. *S. B. naturforsch. Ges. Halle.* 1887. 40—49.

BRAMWELL, J. C. and HILL, A. V., The formation of "breakers" in the transmission of the pulse wave. *J. Physiol. (Lond.)* 1923. *57.* Proc. LXXIII. LXXIV.

DAWSON, P. M., The lateral blood "pressures" at different points of the arterial tree. *Amer. J. Physiol.* 1906. *15.* 244—256.

FRANK, O., Der Puls in den Arterien. *Z. Biol.* 1905. *46.* 441—453.

FRANK, O., Der arterielle Puls. *S. B. Ges. Morph. Physiol. München.* 1926. 33—54.

FREY, M. v., Die Untersuchung des Pulses. *Springer. Berlin.* 260 pp.

FULLER, J., TAYLOR, B. E., CLAGETT, O. T. and WOOD, E. H., Blood pressure in the aorta during resection, and repair of coarctation of the aorta. *J. Lab. clin. Med.* 1952. *39.* 10—25.

GIBSON, A. H., Hydraulics and its applications. London. 1919. 813 pp.

GRASHEY, H., Die Wellenbewegung elastischer Röhren und der Arterienpuls des Menschen. Leipzig. 1881.

HAMILTON, W. F. and DOW, PH., An experimental study of the standing waves in the pulse propagated through the aorta. *Amer. J. Physiol.* 1939. *125.* 48—59.

HARDUNG, V., Zur mathematischen Behandlung der Dämpfung und Reflexion der Pulswellen. *Arch. KreislForsch.* 1952. *18.* 167—172.

HOOBWEG, J. L., Über die Blutbewegung in den menschlichen Arterien. *Pflügers Arch. ges. Physiol.* 1889. *46.* 115—188 and 1890. *47.* 439—453.

HÜRTHLE, K., Experimentelle Beeinflussung der Höhen und Formen des Carotis- und Cruralis-Pulses. *Pflügers Arch. ges. Physiol.* 1934 a. *233.* 262—310.

HÜRTHLE, K., Über die Wirkung der Cocainlähmung der Wand der Bauchaorta sowie der Ausschaltung der Nebennieren auf Blutdruck und Cruralispuls. *Pflügers Arch. ges. Physiol.* 1934 b. *234.* 550—561.

HÜRTHLE, K., Gestaltung und Wirkung des Arterienpulses. *Arch. KreislForsch.* 1944. *14.* 96—154.

KRIES, J. v., Studien zur Pulslehre. *Freiburg Br.* 1892. 146 pp.

KROEKER, E. J. and WOOD, E. H., Comparison of simultaneously recorded central and peripheral arterial pressure pulses during rest, exercise and tilted position in man. *Circ. Res.* 1955. *3.* 623—632.

LANDOIS, L., Die Lehre vom Arterienpuls. Berlin. 1872. 360 pp.
LASZT, L. and MÜLLER, ALOYS, Gleichzeitige Druckmessung in der Aorta abdominalis und ihren Hauptästen. *Helv. physiol. pharmacol. Acta.* 1952. *10.* 259—272.
MALCOLM, J. E., Blood pressure sounds and their meanings. London. 1957. 93 pp.
MÜLLER, ALOYS, Einführung in die Mechanik des Kreislaufes. Abderhalden: *Handbuch der biologischen Arbeitsmethoden.* Wien. 1935. Abt. V. Teil 8. 1—236.
MÜLLER, ALOYS, Über die Grundgesetze der Hämodynamik. *Comptes rendus du IIe Congrès International d'Angéiologie.* Fribourg Suisse 1955. 1—10.
PETERSON, LYSLE H., The dynamics of pulsatile blood flow. *Circ. Res.* 1954. *2.* 127—139.
SCHNABEL, T. G., FITZPATRIC, H. F., PETERSON, LYSLE H., RASHKIND, TALLEY and RAPHAEL, A technic of vascular catheterization with small plastic catheters. *Circulation (N. Y.)* 1952. *5.* 257—262.
SPENGLER, L., Symbolae ad theoriam de sanguinis arteriosi flumine. Marburg. 1843. 39 pp.
TWEEL, L. H. VAN DER, Some physical aspects of blood pressure, pulse wave, and blood pressure measurements. *Amer. Heart J.* 1957. *53.* 4—17.
VOLKMANN, A. W., Die Hämodynamik nach Versuchen. Leipzig 1850. 483 pp.
WEBER, E. H., Über die Anwendung der Wellenlehre auf die Lehre vom Kreislaufe des Blutes und insbesondere auf die Pulslehre. *Arch. Anat. Physiol. wiss. Med.* 1851. 497—546.
WEHN, P. S., Pulsatory activity of peripheral arteries. *Scand. J. clin. Lab. Invest.* 1957. *9.* Suppl. 30.
WEZLER, K. and BÖGER, A., Die Begrenzung des arteriellen Windkessels beim Menschen. *Z. KreislForsch.* 1936. *28.* 391—408.

Acta physiol. scand. 1959. *46*. 119—132.

From the Department of Physiology, Veterinärhögskolan, Stockholm 51, Sweden.

The Response Pattern of Taste Fibres in the Chorda Tympani of the Monkey.

By

GEORGE GORDON[1], RALPH KITCHELL[2], LENNART STRÖM and YNGVE ZOTTERMAN.

Received 16 December 1958.

Abstract.

GORDON, G., R. KITCHELL, L. STRÖM and Y. ZOTTERMAN. The response pattern of taste fibres in the chorda tympani of the monkey. *Acta physiol. scand.* 1959. *46*. 119—132. — The electrical responses of taste fibres in the chorda tympani of Macacus rhesus were recorded during the application of various sapid substances to the tongue. Integrated responses from the whole nerve indicate that this monkey possesses taste fibres responding both to the four conventional classes of taste substances NaCl, acid, quinine and sugar, and also positively to pure water. Records from few-fibre and single-fibre preparations showed that each fibre has a specific pattern of sensitivity to the various sapid substances. Certain gustatory fibres responded very specifically to one class of substances only *e. g.* to salt, acid or to quinine. Fibres responding to sucrose almost always responded to saccharine as well, sometimes also to water, and occasionally to other sapid substances which do not taste sweet to humans, such as quinine or acid. The monkey

[1] On leave from the University Laboratory of Physiology, Oxford; and receiving a Wellcome Research Travel Grant.

[2] Special Research Fellow, U. S. Public Health Service; on leave from Division of Veterinary Anatomy, University of Minnesota, St. Paul 1.

seems to be the first animal investigated possessing taste fibres responding to sucrose which also respond to saccharine. The least specific taste fibres in this animal seem to be those responding to water.

In recent years the response pattern of the whole chorda tympani nerve and also of its individual fibres has been studied in a number of different mammals; and these investigations have shown great species differences. These differences are particularly clear in the response to certain substances which we describe as tasting sweet, like sugars and saccharine. The same is true of the response to pure water: in many animals this is strongly positive, although it is negative in the rat.

The present investigation of the rhesus monkey was undertaken in order to study the electrical response of the chorda tympani in an animal more closely related to man, although at that time we had no information about the direct response from human taste fibres. Some recent attempts to record the integrated response from the human chorda tympani have been successful, however, and have given us a rough idea about the response pattern. One definite finding was that water had no stimulating effect on the taste fibres of the human chorda tympani, but on the contrary lowered or abolished their spontaneous activity (DIAMANT and ZOTTERMAN 1959).

Methods.

Experiments were carried out on eight monkeys (Macacus rhesus), anaesthetised by intraperitoneal injection of 6 per cent, "Mebumal-natrium" solution containing 1.8 g Pentobarbitone, 4 g Pentobarbitone sodium and 25 g urethane in 100 ml. The injected dose was 0.4 ml of this solution per kg body weight. The operation for exposing the chorda tympani differs little from that in the cat (see ZOTTERMAN 1936), although particular care must be taken to avoid penetrating into the mouth through the monkey's cheek-pouch. The chorda was cut as near as possible to its point of entry into the bony channel, and about 5—8 mm of the nerve was usually available for dissection and recording.

The experimental procedure was as follows. The wound was filled with paraffin oil at 37° C, and the nerve lifted on to a platinum electrode attached to a micromanipulator and connected to one input of a differential RC coupled amplifier. The other input, at earth potential, was attached to the tissues exposed in the wound. Responses of the whole nerve to the application of various substances to the tongue were then recorded, using an electronic integrating device which has been described in earlier papers. The sheath was then dissected off and

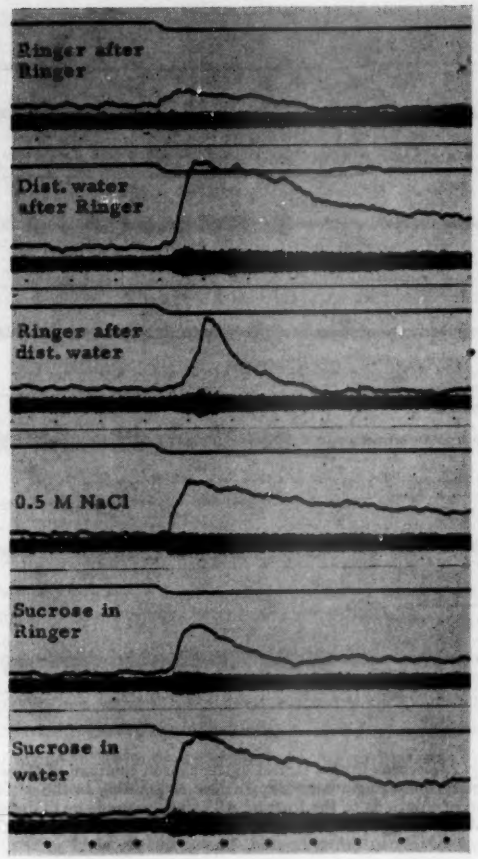


Fig. 1. Electrical responses from the whole chorda tympani of Macacus rhesus to the application of various solutions to the tongue. In each tracing are recorded, from top to bottom: the signal showing the moment of application, the integrated response, and the direct spike response. Time in seconds.

the nerve split into small bundles, from which fine strands were separated by further dissection under a binocular microscope. Single-fibre or few-fibre preparations obtained in this way were tested by applying solutions to the tongue.

All the fibre-preparations were tested with distilled water, Ringer's

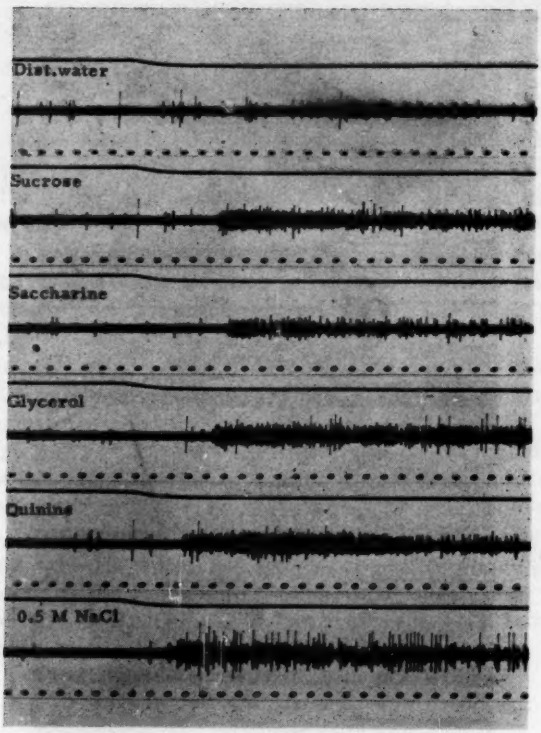


Fig. 2. Records from a strand of the chorda tympani of Macacus rhesus containing a number of fibres responding to the application of solutions to the tongue. All solutions made up in distilled water. A signal shows the moment of application, in this and in other figures. Times: 10 per sec.

solution, and sodium chloride solution (0.5 M); and also with acetic acid (0.2 M), sucrose (0.4 M) and quinine hydrochloride (0.02 M), dissolved either in water or Ringer's solution according to the presence or absence of a response to water. Many fibres were also tested with solutions of saccharine (0.002 M), glycerol (1.4 M), and ethylene glycol (1.8 M), all of which taste sweet to human beings. Fibres responding to salt were tested with a range of sodium chloride solutions (0.002—1 M). All the test solutions were kept in a water bath at about 37° C. The solution was applied by running it on to the tongue through a specially made burette incorporating a signalling device.

Results.

Integrated responses.

On recording the integrated response from the entire chorda tympani, a clear-cut positive response was produced by the application of water to the tongue (see Fig. 1) in seven out of eight monkeys. They all responded to salt, sucrose, saccharine and quinine. In all the nerve preparations tested with these substances, acid, glycerol and ethylene glycol also elicited a positive response.

The effect of Ringer's solution depended upon the prevailing salt concentration on the tongue. If the tongue had previously been rinsed with Ringer's solution the response was quite small, as in Fig. 1, or insignificant: on the other hand it produced a very marked though transient response after a previous rinse with distilled water (Fig. 1).

The effect of watery solutions of non-electrolytic substances like sucrose, in taste nerves which like these contain water fibres, will be a composite response to sucrose and water. Thus 0.4 M sucrose in water will give a much bigger integrated response than 0.4 M sucrose in Ringer's solution. The fact that 0.4 M sucrose in water does not give a much bigger response than water alone could result from sucrose at this concentration partly inhibiting the response of the water fibres. But we may well have to reckon with another factor, namely the viscosity of the sucrose solution, since an increase in viscosity reduces the effect produced on the taste buds by water (LILJESTRAND and ZOTTERMAN 1957). A third possibility is that water and sucrose in some cases stimulate the same taste fibres but to different degrees; and this could produce a certain amount of occlusion of the less vigorous response.

Even assuming that precautions have been taken to avoid any stimulation of thermal fibres or of the rather small number of tactile fibres running in the chorda tympani, the integrated response can, of course, still give only a rough idea of the potency with which a substance stimulates taste fibres. The magnitude of the integrated response is influenced not only by the number of active fibres and their impulse frequencies but also by the size and configuration of the individual spike potentials. So long as we lack detailed information about the dimensions of the different classes of taste fibres there is some need to be careful in assessing the relative responses to different stimuli and in making quantitative comparisons between different species.

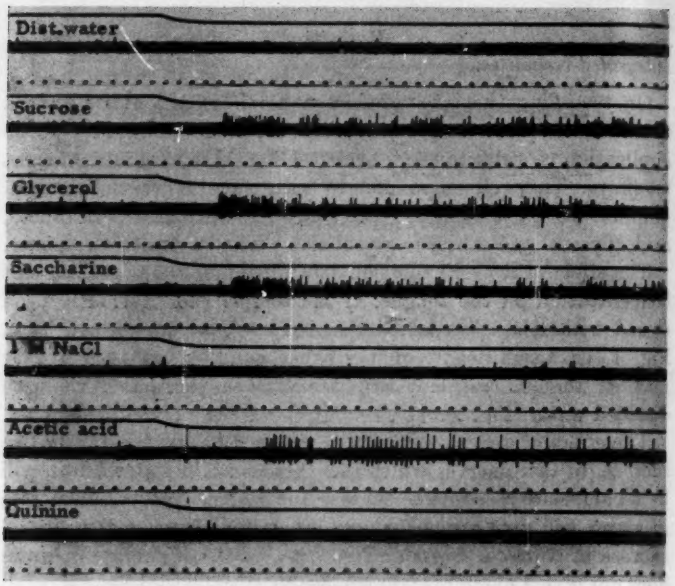


Fig. 3. Records from the same strand of the chorda tympani as in Fig. 2, after reduction in its size by further dissection. Time: 10 per sec.

Responses from individual taste fibres.

General. Records from a fairly fine strand of the chorda tympani are reproduced in Fig. 2. In this preparation there was a persistent response of large spikes at a rather low frequency after a previous rinse with Ringer's solution. The application of water to the tongue abolished this large-fibre response and at the same time gave rise to the appearance of small spikes. Small spikes were produced by all the different sapid solutions used, but large spikes were only elicited by salt in this preparation.

After further reduction of the number of fibres in this preparation quite a different picture was seen (Fig. 3). Neither water, NaCl nor quinine now had any effect. Sucrose, glycerol and saccharine still produced vigorous responses. So also did acid, but in quite another fibre which responded to no other stimulus; and it will be seen from Fig. 3 that this was a single fibre producing spikes of much greater amplitude. We can therefore conclude from this experiment that the

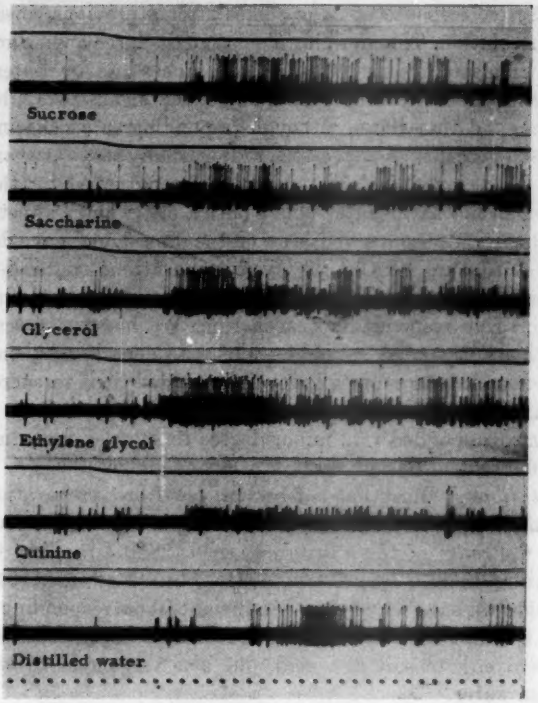


Fig. 4. Records from a small strand of the chorda tympani of Macacus rhesus which contained few active fibres. Note particularly the large spikes, and also those of intermediate size which project both above and below the baseline. All solutions made up in Ringer's solution. Time: 10 per sec.

monkey possessed 1) fibres responding specifically to sweet-tasting substances, 2) fibres responding specifically to salt and 3) fibres responding only to acid. Such preparations, in which for some reason one fibre gives larger spikes than other fibres responding to different stimuli, are very useful in analysis.

"*Sweet fibres.*" The fibre response from another very thin preparation will be seen in Fig. 4. In this case sucrose in Ringer gave rise almost exclusively to large spikes, saccharine, glycerol and glycol elicited both large and small spikes; while quinine gave only small spikes. Acid gave no response at all, while water gave a

very massive response of large spikes after a previous rinse with Ringer's solution. Therefore it seems that the large spikes arose in fibres stimulated by sweet-tasting substances and by water. The fact that quinine in Ringer stimulated the small-spike fibres in this case, and that these, although responding to saccharine, did not respond to sucrose, suggest that functionally they serve another quality than sweet. This possibility is strengthened by the fact that in man saccharine often elicits both a bitter and a sweet taste. Two other sucrose-sensitive fibres, on the other hand, gave definite responses to quinine. Out of seven preparations containing one prominent fibre responding to sucrose, two responded to water as well, and five responded to saccharine. We have only seen one fibre which responded to sucrose but not to saccharine; but that fibre responded far more vigorously to water than to sucrose. It could therefore be more properly classified as a "water fibre" which also responded to a minor degree to sucrose: unfortunately it was not tested with glycerol or ethylene glycol.

"Salt fibres." Most single-fibre or few-fibre preparations responding to salt did so with a quite high degree of specificity within the range of test substances used. "Salt fibres" in the cat respond to acid (PFAFFMANN 1941, COHEN *et al.* 1954); but altogether we have found that out of eight fibres responding to salt in the monkey only four responded to acid. Of these fibres responding both to salt and acid, one also responded to ethylene glycol in water.

It is of course always difficult to be quite confident that such preparations contain one functioning fibre only, but in a case like this where there is no response to acid or any other test solution, specificity of the fibre or fibres to salt is very obvious. The large spikes in Fig. 5 are from such a specific "salt fibre".

In three cases in which we tested with sodium acetate this was found to stimulate "salt fibres", though with a rather higher threshold than for NaCl solutions. In one case we found a "salt fibre" giving a small response to quinine dissolved in distilled water, and in two cases there was a clear response to glycerol in water. One preparation, exceptional in our experience, gave strong responses to the sweet test solutions, a slight delayed response to quinine and to 0.5 M NaCl, and a rather questionable response to 0.2 M acetic acid.

The responses of three "salt fibres" were determined with varying NaCl concentrations. These experiments are shown

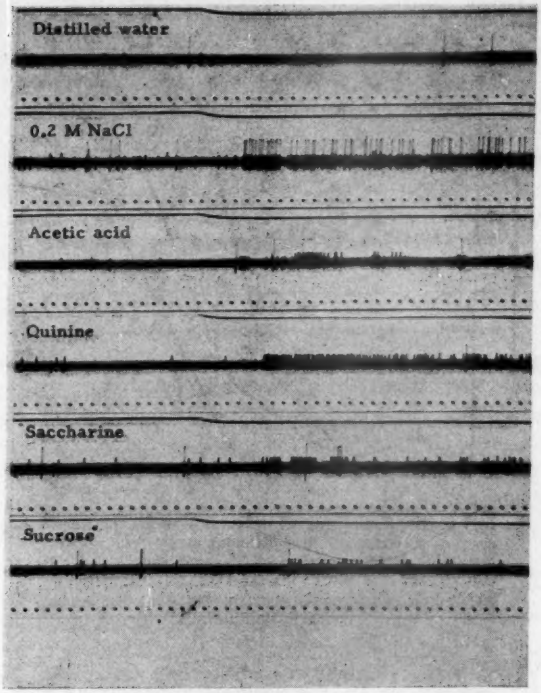


Fig. 5. Records from a small strand of the chorda tympani of Macacus rhesus containing few active fibres. Note particularly the response of large spikes to NaCl, and that of intermediate-sized spikes (projecting principally above the baseline) to other substances, especially quinine. Time: 10 per sec.

graphically in Fig. 6. The threshold varied from less than 0.005 M to somewhere around 0.02 M. The maximum response seemed to be reached at about 1.0 M NaCl.

"*Acid fibres*". Out of seven preparations which gave positive responses to 0.2 M acetic acid only one was strictly specific, *i. e.* did not respond to any other test solution (see Fig. 3). In all the other cases the fibre responded either to quinine, salt, or water, and in two cases even slightly to sucrose.

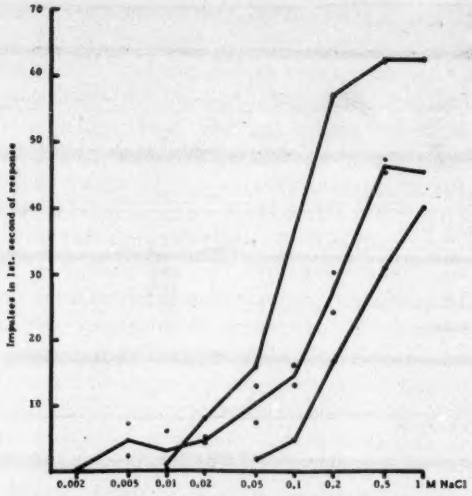


Fig. 6. Graphs showing the relation between the frequency of response of three different single "salt fibres" from the chorda tympani of Macacus rhesus and the concentration of NaCl. Ordinate: number of spikes fired in the first second of the the response. Abscissae: molar concentration of NaCl.

"Quinine fibres". We have found only one fibre which responded exclusively to quinine. Of the other fibres responding to quinine two responded to sucrose; and one of these responded also to saccharine, glycerol, ethylene glycol and NaCl. One fibre responded only to quinine and salt. Finally one fibre (illustrated in Fig. 7) responded strongly to quinine and to acetic acid, and not at all to sucrose or salt. It responded very slightly to water after a previous rinse with Ringer, but after a rinse with 0.5 M NaCl or 0.5 M sodium acetate it gave a vigorous response to water.

Response to water. In spite of the fact that the whole of the nerve generally responded very strongly to the application of water to the tongue we have obtained rather few single-fibre preparations of "water fibres". In the four cases so far studied two responded also to acid and one of these also to quinine (see Fig. 7). One responded only to water and quinine. One fibre responding strongly to water gave equally strong responses to the sweet test solutions (Fig. 4).

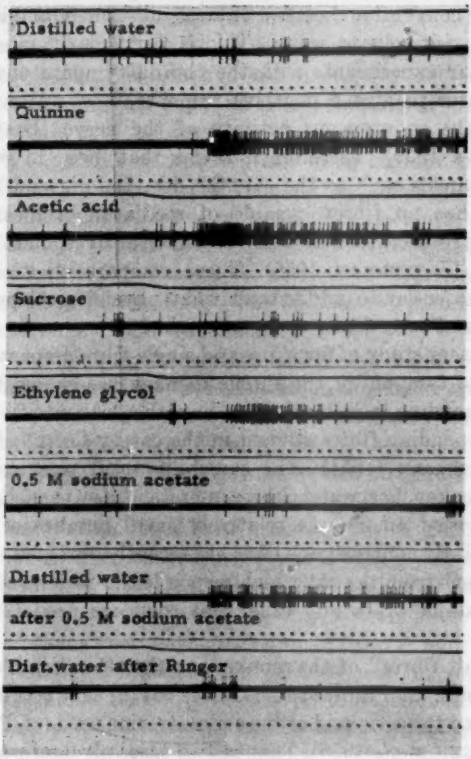


Fig. 7. Records from a small strand of the chorda tympani of Macacus rhesus, containing a single active fibre which responded to quinine, acetic acid, ethylene glycol, and water. It did not respond to NaCl or sodium acetate; but a much increased response to water followed a rinse with either of these. The tongue was rinsed with water between observations except where otherwise stated. All solutions made up in distilled water. Time: 10 per sec.

Discussion.

The responses from the whole nerve show that the rhesus monkey possesses fibres in its chorda tympani which respond to the application of a great variety of sapid solutions to its tongue. It appears to have at least as big a range as any mammal so far investigated by these methods. It is clear that this monkey responds

to all four conventional classes of taste substances, and in addition responds positively to water. This is particularly interesting in that similar experiments with the chorda tympani of man have shown water to give a negative response, *i. e.* water merely abolished the spontaneous activity of the nerve (DIAMANT and ZOTTERMAN 1959). Therefore it seems that man, in contrast to other mammals such as the cat, the dog, the pig and the rhesus monkey, has no fibres capable of mediating a "water taste" and in this respect resembles the rat, which also responds negatively to water (ZOTTERMAN 1956). These comparative taste studies make it no easier to understand what physiological purpose the sensitivity of taste fibres to water might serve.

The present study of few-fibre and single-fibre preparations from the chorda tympani of the rhesus monkey has shown that nerve fibres responding to water seem to be rather less specific than were the corresponding fibres studied in the cat by COHEN, HAGIWARA and ZOTTERMAN (1954). The possibility still remains that the rhesus monkey has water fibres more specific than have so far been observed among the relatively small number of fibres investigated. By contrast, one of us has recently obtained single-fibre preparations from the cat which responded to water and acid, but not to quinine or to any other test solutions used (ZOTTERMAN 1958).

The "salt fibres" of the monkey, on the other hand, seem to be more specific than those studied in the cat, in that half of them did not respond to acetic acid at the concentration used (cf. PFAFFMANN 1941, COHEN *et al.* 1955). That in two cases glycerol was found to stimulate "salt fibres" is the less surpring, when both glycerol and ethylene glycol stimulate taste fibres in chickens and pigeons, in which sucrose has no effect (KITCHELL *et al.* 1959). Sodium acetate stimulated the "salt fibres" whether or not they were sensitive to acid, but the threshold appeared to be rather higher than for NaCl.

It is particularly notable that saccharine had a strong positive effect on almost every preparation containing "sweet fibres" — that is, fibres responding strongly to sucrose. It seems that the monkey is the first animal investigated by electrophysiological methods in which this has been demonstrated. It is known that saccharine solutions can stimulate other types of taste fibre in several types of animal: the response to saccharine in the dog, for instance, can only be attributed to the stimulation of "bitter

fibres" responding to quinine (ANDERSSON *et al.* 1950). In some pigeons, but not in all, KITCHELL *et al.* (1959) found a positive response to saccharine although the nerve did not respond at all to sucrose or quinine. BEIDLER (1953) found that "salt fibres" in the rat could respond to saccharine, and suggested that the sodium salt of saccharine behaved like other sodium salts in this respect. KUSANO and SATO (1957) found responses to saccharine in the glossopharyngeal nerve of Rana nigromaculata which had a similar discharge pattern to that produced by NaCl solutions; and saccharine, like NaCl, depressed the activity of "water receptors". The integrated response of the whole chorda tympani shows that some human taste fibres respond very strongly to 0.002 M saccharine (DIAMANT and ZOTTERMAN 1959). We know for certain that saccharine stimulates "sweet fibres" in the monkey and taking subjective evidence into account this is extremely likely to be true in man. Glycerol and ethylene glycol also stimulate the monkey's "sweet fibres" but these substances can elicit rather smaller responses from other types of receptor as well, and this may also be true of saccharine. This is all consistent with the subjective evidence that sucrose, saccharine, glycerol and glycol all have a sweet taste in man but that only the di- and monosaccharides taste purely sweet; just as NaCl is the only salt which tastes purely salt. All the other sweet-tasting substances have in addition some extraneous quality of taste, such as bitterness in the case of saccharine. Just as in the cat (COHEN *et. al.* 1954), fibres exist in the monkey which respond only to quinine and to other substances which taste bitter to man. In addition to these specific "quinine fibres" we have found fibres responding both to quinine and to water and acid, and also fibres responding to quinine, sucrose and other sweet-tasting substances.

The question of classifying the different types of taste fibre is, of course, an extremely difficult one. A primary classification of taste fibres from animals into groups corresponding to the recognizable human qualities of salt, sweet, sour and bitter, although arbitrary, has certain obvious advantages, and has been partly justified by previous experiments on animals. That it is not a perfect classification has already been demonstrated by the discovery of an additional "water taste" in many animals. It has nevertheless been used here; and certain very specific fibres are easily classified within this scheme. Fibres with complicated pattern of sensitivity to different groups of stimuli have, as far as possible,

been fitted into this classification and any obvious overlapping has been pointed out where it was observed. Our investigation has probably been too limited to reveal the full range of fibres with these more complicated patterns; and we feel, therefore, that a more extensive objective classification should await a more detailed investigation. We have the impression that in this respect the situation is more complicated in the monkey than in, for instance, the cat.

Without knowing anything of the taste sensations which monkeys actually experience, we can say, that this animal has a range of chemical sensitivity in its tongue which seems to agree with what one would expect from an animal phylogenetically so close to man. Even so, a great deal of caution is necessary. The work of PFAFFMANN and BEIDLER, as well as all previous investigations made in our laboratory, have given abundant evidence that there are numerous species differences in the peripheral nervous mechanism of taste, and in the present case the monkey's positive response to water points to one striking difference between monkey and man.

References.

ANDERSSON, B., S. LANDGREN, L. OLSSON and Y. ZOTTERMAN, The sweet taste fibres of the dog. *Acta physiol. scand.* 1950. *21.* 105—119.

BEIDLER, L. M., Properties of chemoreceptors of tongue of rat. *J. Neurophysiol.* 1953. *16.* 595—607.

COHEN, M. J., S. HAGIWARA and Y. ZOTTERMAN, The response spectrum of taste fibres in the cat: A single fibre analysis. *Acta physiol. scand.* 1955. *33.* 316—332.

DIAMANT, H. and Y. ZOTTERMAN, Has water a specific taste? *Nature* (*Lond.*) 1959. *183.* 191—192.

KITCHELL, R., L. STRÖM and Y. ZOTTERMAN, Electrophysiological studies of thermal and taste reception in chickens and pigeons. *Acta physiol. scand.* 1959. In press.

KUSANO, K. and M. SATO. Properties of fungiform papillae in frog's tongue. *Nippon Seirigatsee Jassi* 1957. *7.* 324—338.

LILJESTRAND, G. and Y. ZOTTERMAN, The effect of certain demulcents on the taste nerve endings. Festschrift Arthur Stoll. *Sandoz A. G. Basel.* 1957. 806—813.

PFAFFMANN, C., Gustatory afferent impulses. *J. cell. comp. Physiol.* 1941. *17.* 243

ZOTTERMAN, Y., Specific action potentials in the lingual nerve of cat. *Skand. Arch. Physiol.* 1936. *75.* 105—119.

ZOTTERMAN, Y. Species differences in the water taste. *Acta physiol. scand.* 1956. *37.* 60—70.

ZOTTERMAN, Y. Studies in the nervous mechanism of taste. *Exp. Cell. Res.* 1958. Suppl. 5. p. 520—526.

Acta physiol. scand. 1959. *46*. 133—151.

From the Department of Physiology, Veterinärhögskolan, Stockholm 51, Sweden.

Electrophysiological Studies of Thermal and Taste Reception in Chickens and Pigeons.

By

R. L. KITCHELL[1], LENNART STRÖM and YNGVE ZOTTERMAN.

Received 16 December 1958.

Abstract.

KITCHELL, R. L., L. STRÖM and Y. ZOTTERMAN. — Electrophysiological studies of thermal and taste reception in chickens and pigeons. *Acta physiol. scand.* 1959. *46*. 133—151. — The impulse traffic in thermal and taste nerve fibres from the tongue of chickens and pigeons were analyzed while applying adequate stimuli to the tongue. Receptors were found in the tongue and pharynx which respond, in general, like those found in mammals. The thermal and taste receptors discharge through two peripheral branches of the 9th nerve only. In the chicken positive responses from the nerves followed the application of distilled water, salt, glycerine, ethylene glycol, quinine and acetic acid to the tongue but not of sucrose and saccharine. The taste responses in the pigeon's nerves were similar with the exception that no responses were observed to quinine and that 50 per cent of the pigeons responded positively to saccharine although sucrose was ineffective. In both species large number of "cold fibres" were observed while warming the tongue gave no response until the temperature was

[1] Special Research Fellow, Division of Neurological Diseases and Blindness, U. S. Public Health Service on leave from Division of Veterinary Anatomy, College of Veterinary Medicine, University of Minnesota, St. Paul 1, Minnesota.

raised to above 45° C. This indicated the absence of "warm fibres" and suggested that the activity observed was due to stimulation of "pain fibres". These direct studies of taste nerve activity were compared with recent behavioural studies in chickens and pigeons.

Taste in birds has been a widely discussed problem. Many investigators have maintained that birds have no taste or that taste in these animals should be rudimentary. Taste buds have, however, been described in various birds by BOTEZAT (1904, 1906) by BATH (1906) and by MOORE and ELLIOTT (1946) among others. Also a number of behavioural studies have been conducted which indicate that birds prefer certain substances while they reject others (ENGELMANN 1934, 1937 a and b, 1940, JACOBS and SCOTT 1957, KARE *et al.* 1957). In contrast to numerous reports on neurophysiological studies on taste in other animals (*e. g.* ZOTTERMAN 1936, 1956, PFAFFMANN 1955, BEIDLER *et al.* 1955) no reports have appeared describing such studies in birds. The purpose of this report is to present the results of such a study which indicate that birds, in the innervation of the posterior part of the tongue and the pharynx, possess afferent fibres, which respond specifically to the application of sapid substances to their endings.

Materials and Methods.

A total of 18 chickens and 15 pigeons were used in this study. The chickens were adult hens of the White Leghorn breed. The pigeons were wild native birds, undoubtedly of mixed breeding.

In the initial stages of the study a number of anaesthetics were tried with considerable mortality occurring, apparently due to respiratory failure. Numal (Roche) administered via a cannulated wing vein, was used in the majority of the pigeons and in a few of the chickens. Finally, the most successful anaesthetic was found to be urethane administered intravenously as a 43 per cent solution (1.5 g per kg) as described by KING and BIGGS (1957). Artificial respiration was given via tracheal cannula throughout the experiment. During recording, d-tubocurarine (0.75 mg/kg in the pigeon and 3 mg/kg in the chicken) was administered intravenously to abolish the swallowing reflex.

The surgical procedure consisted of making a skin incision parallel to the ventral border of the right ramus of the mandible. After doubly ligating and incising the branches of the external maxillary blood vessels, the muscles were transected to expose the nerves to the right side of the tongue. The exposed nerves were dissected free and cut near their origin from the parent nerve. The right ramus of the mandible

was transected near the right angle of the mouth and the distal portion was pulled laterally to expose the tongue more fully. The mandible was anchored firmly to a cork by means of pins. A suture was placed through the left side of the tongue and was used to pull the tongue forward. A special burette was then fixed in position with its tip approximately 5 mm above the tongue and so that the solutions would flow over the portions of the tongue predetermined to be sensitive to touch. A switch was attached to the stopcock of the burette which signalled on the record when the solution was released from the burette. Five ml quantities of test solution were used for each trial and the burette and the tongue were flushed with Ringer's solution between each application unless otherwise indicated. In order to avoid any stimulation of thermal receptors, the sapid solutions were kept in a water bath at an average temperature of 39° C.

The further preparation of the nerves and the splitting up into fine strands was performed according to the method previously described by ZOTTERMAN (1936). The action potentials were lead off through Ringer-soaked, cotton electrodes, amplified by a differential amplifier of the Tönnies type and recorded on moving paper by means of a cathode ray oscillograph.

The thermode used was similar in design to that described previously by HENSEL *et al.* (1951).

Results.

Innervation of the tongue. In birds it is difficult to demarcate the posterior border of the tongue from the floor of the pharynx. We have adopted the terminology of MOORE and ELLIOTT (1946) who considered that the tongue fold separated the anterior portion from the posterior portion, centrally, and the tongue wings laterally. In keeping with the observations of OWEN (1866) and KAUPP (1918) we could find no branches from the trigeminal nerve going to the tongue. We were unable to record any responses in that nerve following the application of cold, touch or various "taste" solutions to the tongue. The nerves to the tongue were found to arise from the glossopharyngeal nerve. KAUPP (1918) described two branches of the glossopharyngeal nerve going to the tongue. He referred to the more anterior of these as the recurrent lingualis nerve which he stated supplied the tongue and the pharynx. The more posterior nerve he called the recurrent pharyngeus nerve and described it as innervating the base of the tongue, the pharynx and the esophagus. Our correlated anatomical and neurophysiological findings do not agree with his anatomical description. We have found two distinct nerves, arising independently from the glossopharyngeal nerve which go

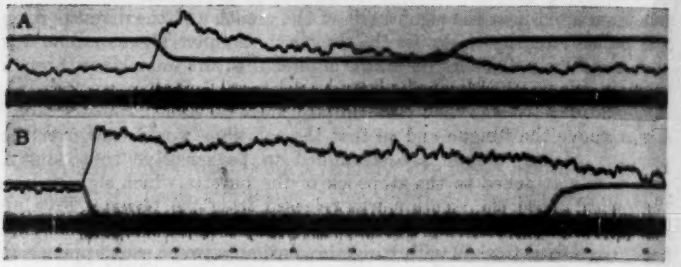


Fig. 1. Records from the lingual nerve of the chicken showing the responses to sudden cooling and rewarming the tongue. In each are recorded, from top to bottom: the temperature of the tongue, the integrated response, and the direct spike response. Time marks, 1 cps.

A: 39—36° C B: 48—35° C.

to the tongue instead of the single recurrent lingualis nerve described by him. We did not find any branches from the recurrent pharyngeus nerve, more appropriately called pharyngo-esophageal nerve, going to the tongue.

The anterior nerve to the tongue, referred hereafter as the lingual nerve, arises from the glossopharyngeal nerve and runs ventrally, just anterior to the lingual artery. It soon separates from the artery to run above the lingual bone and enter the tongue. It gives off a few small branches to the lateral wall of the pharynx but mainly goes to the anterior portion of the tongue, part of the wings and parts of the posterior portion of the tongue. The lingual nerve of the pigeon is much smaller than that of the chicken.

The posterior nerve, referred hereafter as the laryngo-lingual nerve, arises from the glossopharyngeal nerve distal to the origin of the lingual nerve. It runs ventrally, just posterior to the lingual artery giving a few branches to the lateral wall of the pharynx. It is coiled in its proximal portions, especially in the chicken. The laryngo-lingual nerve runs lateral to the larynx, giving branches to its internal and external surfaces, then terminates in the posterior portion and wings of the tongue. Both the lingual and laryngo-lingual nerves supplied the posterior portion and wings of the tongue.

Temperature, chicken: In experiments conducted at room temperature (20° C) both the lingual and the laryngo-lingual nerves showed a constant high level of spontaneous activity. This activity was so great that it masked the effects of the application

of test solution (at 39° C) to the tongue. Warming the tongue to a constant temperature of 38—40° C by means of a thermode greatly reduced, but did not entirely eliminate, the spontaneous activity (Fig. 1 A). A sudden cooling of the tongue as little as 3° C resulted in an abrupt increase in the activity in these nerves. This initial response lasted for about 2 sec, following which the electrical activity gradually declined to a level slightly higher than the precooling level. Rewarming to the tongue to its original level resulted in a reduction of the electrical activity to the precooling level. The responsiveness of these fibres to small drops in temperature indicates that there are specific "cold" receptors in the tongue of chickens (see HENSEL and ZOTTERMAN 1950).

A larger temperature drop, even from a higher initial temperature, resulted in a greater discharge (Fig. 1 B). The electrical activity did not decline as rapidly as when smaller temperature drops were applied. Rapid rewarming of the tongue did not result in a rapid return of the electrical activity to its precooling level. Instead, it continued at a high level for 3—4 sec after rewarming before it returned to the original level. The presence of a high level of electrical activity after rewarming the tongue suggested that the rewarming stimulated either "warm fibres" or "pain fibres". This phenomenon was not observed to occur if the final temperature was kept below 44° C (Fig. 1 A) indicating the absence of warm fibre activity (DODT and ZOTTERMAN 1952 a).

The effect of temperature on the tongue receptors was examined more closely using thin strands of the lingual nerve where only a few fibres were active (Fig. 2, 3 and 4). Maintaining the temperature of the tongue at 44° C resulted in a steady state frequency in a single "cold fibre" of 2—4 imp/sec (Fig. 3). A temperature drop of 9° C (Fig. 2 A and 3) stimulated an initial response of 29 imp/sec which gradually declined for about 10 sec to a new steady state frequency of 8—10 imp/sec which it will maintain for several minutes. Rewarming the tongue will result in a cessation of activity in the "cold fibre" for several seconds and cause the appearance of spikes of lower height (Fig. 2 B). These smaller spikes, which apparently are produced by fibres other than the "cold fibres", appear only when the temperature of the tongue is raised above 44° C, which indicates that they should be classified as "pain fibres" (cf ZOTTERMAN 1936). In experiments where the tongue was repeatedly warmed and cooled, it was seen that the first application of heat produces a greater initial response

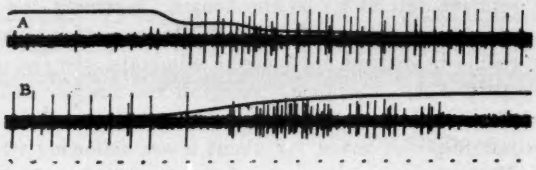


Fig. 2. Action potentials from a thin strand of the chicken's lingual nerve containing a single "cold fibre" and several "pain fibres". The thin line shows the temperature of the tongue. Time marks, 10 cps.

A: 44—35° C B: 35—44° C.

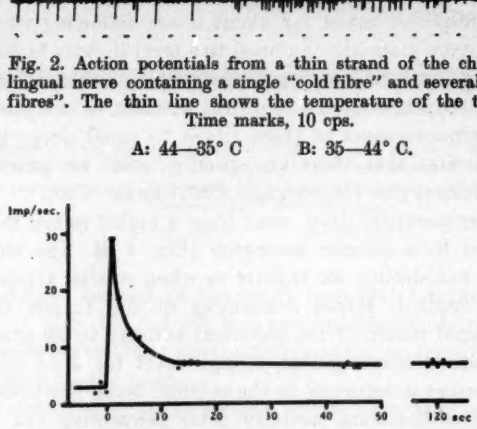


Fig. 3. Graph showing the impulse frequency of a single "cold fibre" in the lingual nerve of the chicken in response to a sudden cooling of the tongue from 44—35° C.

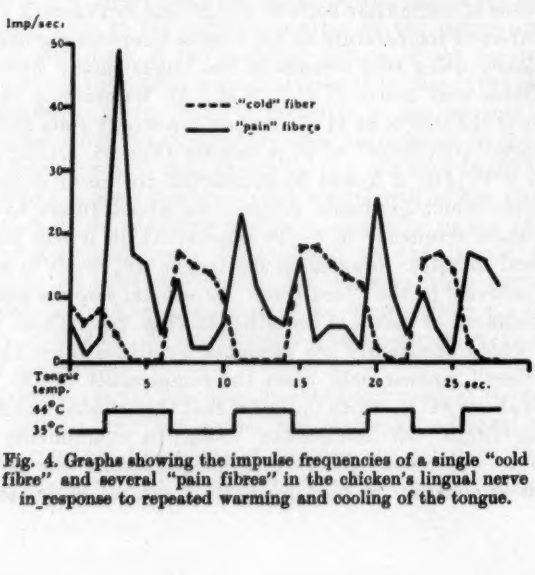


Fig. 4. Graphs showing the impulse frequencies of a single "cold fibre" and several "pain fibres" in the chicken's lingual nerve in response to repeated warming and cooling of the tongue.

than do subsequent applications in that series (Fig. 4). A brief "off" response of the smaller "pain fibres" can also be seen (Fig. 2 A and 4).

The mode of action of the "cold fibres" seems quite similar to those described for the cat by HENSEL and ZOTTERMAN (1952 a) except that the "cold fibres" in the chicken continue to be active at higher constant tongue temperatures than do those in the cat. No response of the specific "cold fibres" in the chicken was observed at higher temperatures (48—54° C), "paradoxical cold", as was observed in the cat by DODT and ZOTTERMAN (1952 b).

Temperature, pigeon: Very little spontaneous activity was present in the lingual nerve of the pigeon. Stepwise cooling the tongue 3—5° did not result in the appearance of a response in this nerve. However, larger temperature drops elicited the appearance of large spikes (Fig. 5 A). These spikes were of the same amplitude and form as those appearing in this nerve following the application of pressure to the anterior portion of the tongue. The form of these spikes, and the parameters of stimulation needed to elicit them, indicate that they are derived from mechanoceptive fibres stimulated by cooling as described by HENSEL and ZOTTERMAN (1951).

The laryngo-lingual nerve exhibited more spontaneous activity than did the lingual nerve. Stepwise cooling the tongue resulted in the appearance of responses in this nerve (Fig. 5 B—C). No response was obtained to changes from 42° to 38° C (Fig. 5 D) and only brief phasic responses were stimulated by changes from 36 to 32° C (Fig. 5 C). These responses indicate that the "cold fibres" in the pigeon are inactive at tongue temperatures which, in the chicken, would result in a steady discharge of "cold" impulses. A positive response was elicited by warming the tongue above 45° C indicating the presence of "pain fibres" (Fig. 5 E). No "warm fibres" were found in these nerves.

Taste, pigeon: The lingual nerve gave more pronounced responses to the application of sapid solutions to the tongue than did the laryngo-lingual nerve. This may presumably be due to the absence of "cold fibres" in the lingual nerve with the resultant improvement in the signal-to-noise ratio.

In each trial the tongue was washed with Ringer's solution before the test solution was applied. The results of the application of sodium chloride solutions of different strengths, from 0.5 M to distilled water, is shown in Fig. 6. It can be seen that 0.5 M

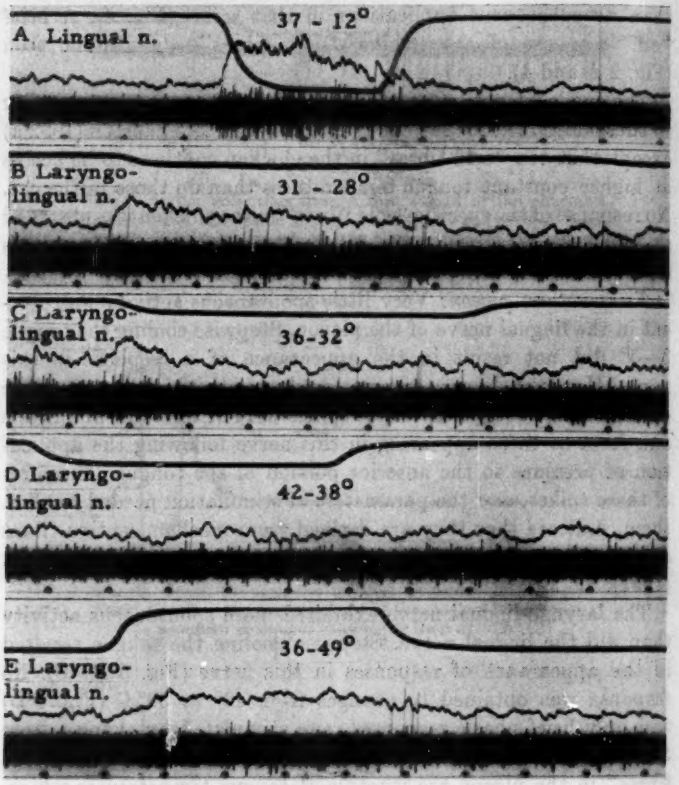


Fig. 5. Temperature responses in the pigeon. The recording technique is the same as in Fig. 1. Time marks, 1 cps.

NaCl elicits a distinct response. When the concentration of NaCl was lowered to 0.1 M no response was obtained. Further reducing the concentration results in the reappearance of a response. This response, however, has a longer latency and duration than the response produced by 0.5 M NaCl. The response to salt solutions having concentrations of 0.05 M or less is similar in latency, magnitude and duration to that produced by distilled water. Similar responses were obtained using choline chloride and sodium acetate. The rinsing of the tongue with distilled water, instead of Ringer's solution, enhances the response to salty solutions,

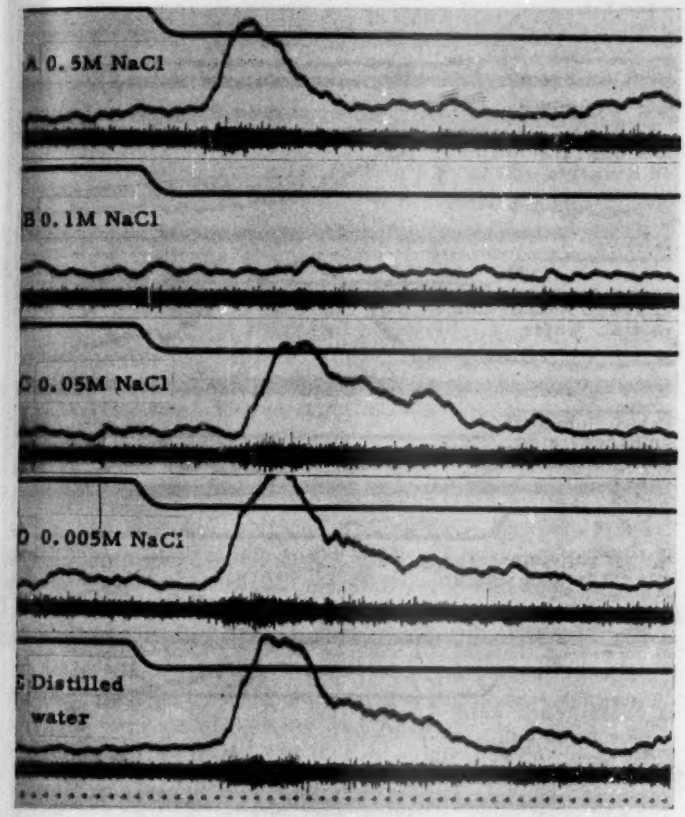


Fig. 6. Records from the pigeon's lingual nerve during application of NaCl solutions of different concentrations to the tongue. In each are recorded, from top to bottom: the signal from the dispensing burette (downward deflection indicates release of the solution), the integrated response and the direct spike response. Time marks, 10 cps.

thus even Ringer's solution elicited a slight phasic response under these circumstances. These findings indicate that pigeons have receptors located on the tongue which are sensitive to salt concentrations of 0.2 M and above, and to distilled water. These responses were examined more closely using thin strands of the lingual nerve (Fig. 8 A and B). In Fig. 8 A it can be seen that the application of 0.5 M NaCl to the tongue results in the appear-

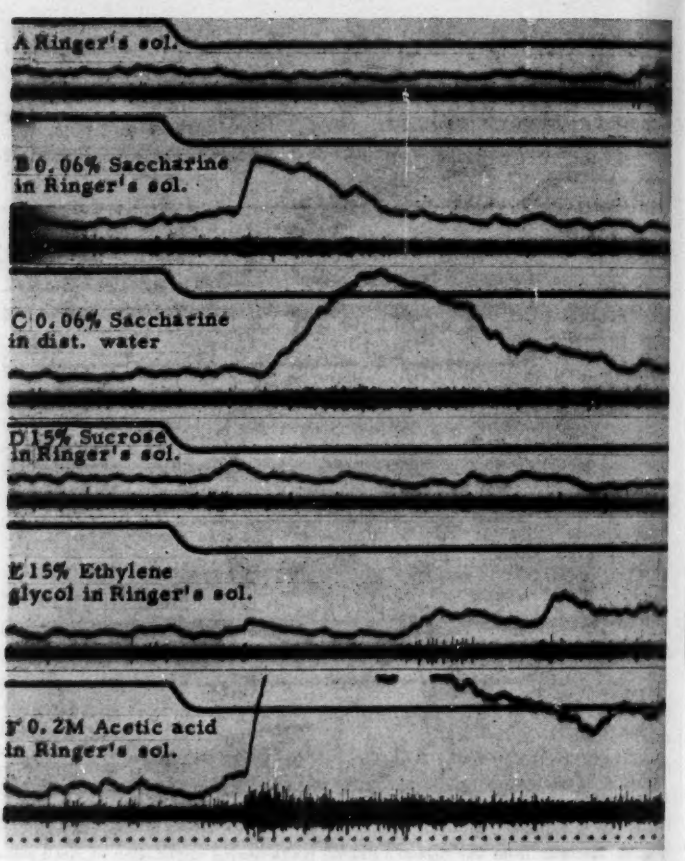


Fig. 7. Records from the pigeon's lingual nerve during application of different solution to the tongue. The recording technique is the same as in Fig. 6. Time marks, 10 cps.

ance of small spikes in the fibres of the lingual nerve. The application of distilled water elicits spikes of much larger amplitude (Fig. 8 B). These observations indicate that the fibres carrying impulses from the tongue in response to salt solutions ("salt fibres") are different from those carrying impulses in response to distilled water ("water fibres"). These findings are in agreement with those of ZOTTERMAN (1949), ANDERSSON and ZOTTER-

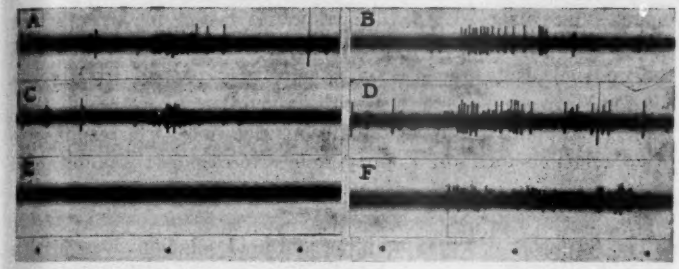


Fig. 8. Action potentials from a small strand of the pigeon's lingual nerve in response to the application of one drop of the following solutions to the tongue. Time marks, 1 cps.

A: 0.5 M NaCl
B: Distilled water
C: 15 % Glycerine in Ringer's sol.
D: 15 % Glycerine in distilled water
E: 0.02 M Quinine in Ringer's sol.
F: 0.2 M Acetic acid in Ringer's sol.

MAN (1950), LILJESTRAND and ZOTTERMAN (1954), COHEN *et al.* (1955) and ZOTTERMAN (1956). The presence of "water fibres" made it imperative to administer all other substances in Ringer's solution to which the "salt" receptors can be more or less completely adapted by a Ringer's solution wash prior to testing (ZOTTERMAN 1956).

The results of testing sweet and acid solutions are illustrated in Fig. 7. In about 50 per cent of the pigeons a response was present to the application of 0.06 per cent saccharine in Ringer's solution (Fig. 7 B). In these birds, 0.06 per cent saccharine in distilled water produced a response which was different from saccharine in Ringer's solution or from distilled water alone (Fig. 6 D). In birds which showed no response to saccharine in Ringer's solution. the application of saccharine in distilled water resulted in a response which could not be distinguished from distilled water alone. Fifteen per cent sucrose in Ringer's solution seldom elicited a response. When it did, the response was always small and questionable (Fig. 7 D). In contrast, 15 per cent sucrose in distilled water always produced a response which was indistinguishable from that produced by distilled water alone. Fifteen per cent ethylene glycol or 15 per cent glycerine (both of which taste sweet to humans) in Ringer's solution, gave responses, starting after a relative long latency of about 1.6 sec after the application (Fig. 7 E). The response gradually increased in intensity to reach

a maximum after 90 sec. The responses lasted for over 2 min and were difficult to stop, even with repeated rinsing of the tongue with Ringer's solution. Acetic acid (0.2 M in Ringer's solution) always produced a strong, long-lasting response (Fig. 7 F). The application of acid solutions depressed the response to other solutions for more than an hour. No response was obtained at any time to 0.02 M quinine hydrochloride in Ringer's solution.

The responses of sweet, quinine and acid solutions was further studied using thin strands of the lingual nerve (Fig. 8 C—F). Glycerine in Ringer's solution initiated activity in fibres having similar spike heights as "salt fibres". Our results do not show whether they are the same fibres or not. Glycerine in distilled water resulted in the appearance of spike potentials of two different heights, one presumably due to the glycerine and the other presumably due to the activity of "water fibres". Quinine and sucrose did not initiate a response in these preparations. Acetic acid stimulated spike potentials slightly higher than glycerine or salt, but less in height than distilled water.

Taste, chicken: To avoid that the stimulation of cold receptors should mask the response of "taste fibres", it was necessary to keep the tongue warm using a heat lamp and to have the temperature of the solutions between 38—40° C. Care had to be taken to remove as much of the sheath around the nerve as possible in order to increase the signal-to-noise ratio so that the small "taste fibre" spikes would appear above the noise level.

Both the lingual and laryngo-lingual nerves gave similar responses. When Ringer's solution was applied to the tongue it was difficult to avoid a small, phasic discharge, presumably due to the stimulation of touch fibres. By careful placement of the tip of the burette this could be eliminated almost completely (Fig. 9 A and 10 A). Distilled water elicited a sustained response for several seconds (Fig. 9 B). Using fine strands of the nerve it was observed that the spikes were considerably smaller than those produced by touch (Fig. 10 B). The application of 0.5 M NaCl solutions produced only moderate responses in some birds (Fig. 9 C) and gradually increasing, sustained responses in others. The effect of 15 per cent sucrose in Ringer was often questionable, the response usually being somewhat larger than the small phasic response produced by Ringer alone (Fig. 9 A and D). Similarly, 0.06 per cent sac-

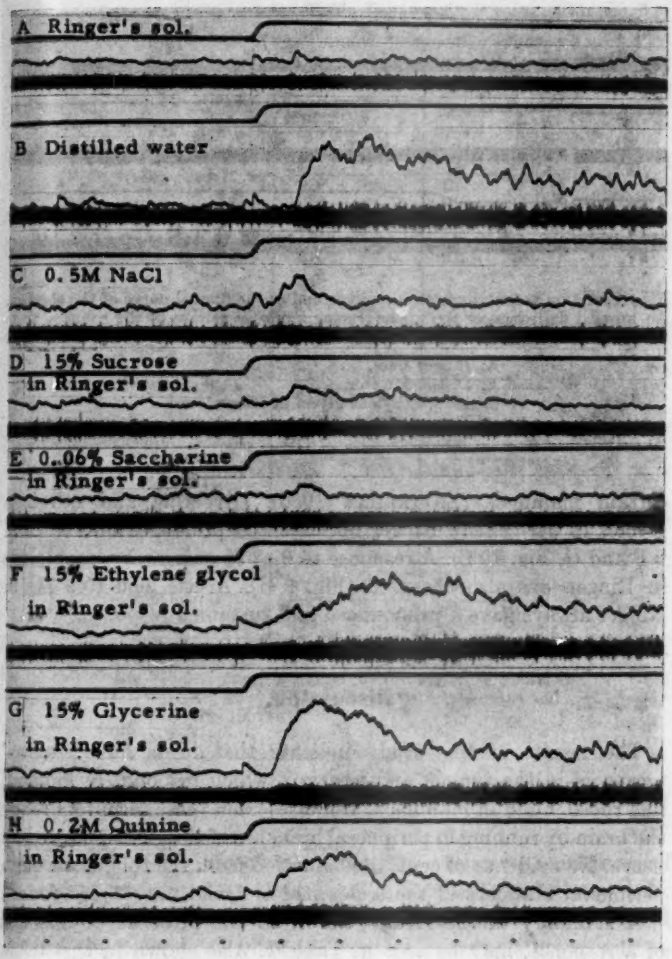


Fig. 9. Records from the chicken's lingual nerve during application of different solutions to the tongue. In each are recorded, from top to bottom: the signal from the dispensing burette (upward deflection indicates release of the solution), the integrated response and the direct spike response. Time marks, 1 cps.

charine in Ringer produced a slightly larger effect than Ringer alone (Fig. 9 E). Neither of these substances produced effects comparable to saccharine in the pigeon (Fig. 6 B) or sucrose in

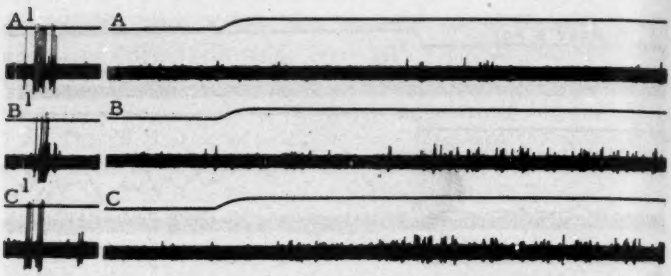


Fig. 10. Action potentials from a small strand of the lingual nerve of the chicken. An upward deflection of the upper tracing indicates release of the solution from the dispensing burette. A^1, B^1, C^1 touch to the tongue 2 sec. before A, B, C.

A: Ringer's sol.
B: Distilled water.
C: 15 % Ethylene glycol in Ringer's sol.
Time marks, 1 cps.

certain mammals (ZOTTERMAN 1956). Glycerine and ethylene glycol, 15 per cent in Ringer both elicited prolonged effects (Fig. 9 F and G, Fig. 10 C). A response to 0.02 M quinine hydrochloride in Ringer was also observed (Fig. 9 H). Acetic acid (0.2 M) in Ringer always gave a pronounced and sustained response.

Discussion.

The results of this study indicate that birds have "taste" receptors in the tongue and pharynx which respond, in general, like those found in mammals. Impulses from these receptors reach the brain by running in peripheral branches of the glossopharyngeal nerve. No evidence of central conduction from the tongue through peripheral branches of the trigeminal or facial nerves was found.

A summary of the results, based on the presence or absence of a response, may be seen in Table I. Also shown in this table is a summary of the results of behavioural studies reported by other workers. In comparing the results of the two different kinds of studies, it must be clearly understood what each kind of study reveals. Our electrophysiological study yields information regarding the peripheral sensory input going from the tongue to the brain. For example, it can determine the presence or absence of a response to the application of a substance to the tongue.

Table I.

Comparison of taste and behavioural response in chickens and pigeons.

	Chicken		Pigeon	
NaCl	TR / BR	+ / R[1,2]	TR / BR	+ / R[1]
Sucrose	TR / BR	— / P[1,2,3]	TR / BR	— / A[1]
Glycerine	TR / BR	+ / R[1]	TR / BR	+ / R[1]
Ethylene glycol ..	TR / BR	+ / 0	TR / BR	+ / 0
Saccharine	TR / BR	— / R[1,2,3]	TR / BR	+ / R[1]
Quinine	TR / BR	+ / R[1,2]	TR / BR	— / A[1]
Acetic acid	TR / BR	+ / R[1]	TR / BR	+ / R[1]

TR — electrical response from nerves;
BR — behavioural response according to [1]) ENGELMANN, 1934 [2]) KARE *et al.*, 1957 [3]) JACOBS and SCOTT, 1957;
R — reject;
A — do not discriminate against;
P — prefer;
0 — not determined.

It can also yield information about the relative sensitivity of the receptors located on the tongue in terms of the latency, size, and duration of the recorded response. Electrophysiological studies do not reveal what the central nervous system will do with the information supplied to it from its receptors. Behavioural studies, on the other hand, do reveal what the central nervous system does in respect to the total sensory input under conditions of the study but will not reveal much information about the nature of the sensory input. Behavioural studies will show whether an animal will prefer, reject or not discriminate for or against a substance, but usually will not demonstrate whether that substance

actually stimulates chemoreceptors on the tongue (taste in its more specific sense), or touch or cold receptors on the tongue, or similar receptors, located elsewhere in the mouth or on prehensile organs. Behavioural studies also have difficulty in separating the input from olfactory receptors from that of taste receptors and eliminating the effect of prior experiences on the behavioural responses observed.

To use the word "taste" at all in relation to the response of an animal must be done anthropomorphically, *i. e.* projecting human sensations into the "minds" of animals. Also the ascribing of such terms as "sweet", "salt", "bitter" and "acid" tastes to animals involves similar reasoning. It is, however, burdensome to add after each statement relative to taste in animals, "as perceived by man". For the purposes of this discussion we will use the terms "taste response" to refer to changes in the input from the chemoreceptors in the tongue and pharynx following the application of a substance which tastes "salt", "sweet", "bitter" or "acid" to human beings. Also, since distilled water has been shown to elicit a specific response in the frog (ZOTTERMAN 1949) and other species of animals (ZOTTERMAN 1956) reference will be made to a specific "water" taste response.

In Table I it can be seen that the chicken has distinct taste responses to salt, bitter, acid and water. The response of the chicken to sweet is of a questionable nature. No definite responses to sucrose or saccharine were obtained in any of the experiments. On the other hand ethylene glycol and glycerine, both of which taste sweet to humans, gave pronounced responses. Other studies have shown considerable species differences in the responses obtained to saccharine. BEIDLER (1953) states that it acts like a salt in the rat. ZOTTERMAN (1956) reported no response to saccharine in the rat, rabbit, pig and cat. The last animal also failed to give a response to sugar. Further studies, particularly using single fibre preparations, will be needed to clarify the response of the chicken to sweet tasting substances.

The pigeon has distinct taste responses to water, salt and acid, no response to bitter, and a questionable response to sweet. Again, like the chicken, responses were obtained to ethylene glycol and glycerine. Unlike the chicken, 50 per cent of the pigeons showed positive responses to saccharine.

In general, a good correlation can be seen between the presence, or absence, of a taste response and the behaviour of the bird

in relation to a particular substance (Table I). In two instances there appears to be a difference in the results. Chickens seem to prefer sucrose solutions over water (ENGELMANN 1934, KARE *et al.* 1957, JACOBS and SCOTT 1957) whereas no response to sucrose was observed in our studies. The degree of preference observed in the behavioural studies was small in all instances. JACOBS and SCOTT (1957) suggested that the preference could be due to a difference in viscosity rather than due to a taste response. The results of behavioural studies indicate that chickens prefer water over saccharine. In our study no taste response to saccharine in Ringer's solution was observed. Saccharine in distilled water produced a response indistinguishable from distilled water alone. Our observations do not suggest the possible sources of the afferent inflow which enables chickens to discriminate against saccharine, other than to state that it probably does not originate from any taste receptors in the tongue.

It would be agreed that it is difficult to use the integrated responses as a means of quantitative comparison of the different taste responses in various species. Such a method has a number of limitations. In the present investigation it was found that water in the pigeon elicits considerable greater response than salt (Fig. 6). To conclude that there were more water receptors than salt receptors on the tongue, or that the difference in response was due to the water receptors responding more strongly than the salt receptors, would disregard the observation that the individual spike potentials elicited by water appear to be much larger than those produced by salt (Fig. 8). This difference in spike height could account for most, if not all, of the difference in the magnitude of the integrated responses following the application of these two substances, as most integrators express not only the number of impulses in a nerve per unit time but also reflect the time integral of the individual spike potentials being recorded. This property of the integrator is of considerable importance as the magnitude of the integrated response is influenced not only by the number of active fibres and their impulse frequency but also by the size and the configuration of the individual spike potential.

In the frog the fibres of the glossopharyngeal nerve which respond specifically to water and to salt respectively are fairly large myelinated fibres (A β) while those responding to bitter are obviously much smaller (Aδ) fibres (ZOTTERMAN 1949, AN-

DERSSON and ZOTTERMAN 1950). Any attempt to compare the relative magnitude of response in birds to that found in other animals would seem to be futile because of the great differences in the nerve supply to the tongue and in the size of the receptive field being stimulated.

The presence of large numbers of cold receptors in the tongue of birds raises a question as to the influence of cold on their preference for certain foods. This is particularly interesting in the chicken where the receptors are still active at constant temperatures significantly higher than those observed in the pigeon and mammals. The absence of warm fibres suggests that the sensory inflow which assists birds in determining the relative warmth of a food may function on the basis of a diminution of cold receptor activity. It is also possible that warm receptors may be present elsewhere in the head region.

References.

ANDERSSON, B. and Y. ZOTTERMAN, The water taste in the frog. *Acta physiol. scand.* 1950. *20*. 95—100.

BATH, W., Die Geschmacksorgane der Vögel und Krokodile. *Arch. Biontologie.* 1906. *1*. 5—47.

BEIDLER, L. M., Properties of chemoreceptors of tongue of rat. *J. Neurophysiol.* 1953. *16*. 595—607.

BIEDLER, L. M., I. Y. FISHMAN and C. W. HARDIMAN, Species differences in taste responses. *Amer. J. Physiol.* 1955. *181*. 235—239.

BOTEZAT, E., Geschmacksorgane und andere nervöse Endapparate im Schnabel der Vögel. *Biol. Zbl.* 1904. *24*. 722—736.

BOTEZAT, E., Die Nervenendapparate in den Mundteilen der Vögel und die einheitliche Endigungsweise der peripheren Nerven bei den Wirbeltieren. *Z. wiss. Zool.* 1906. *84*. 205—360.

COHEN, M. J., S. HAGIWARA and Y. ZOTTERMAN, The response spectrum of taste fibres in the cat: a single fibre analysis. *Acta Physiol. Scand.* 1955. *33*. 316—332.

DODT, E. and Y. ZOTTERMAN, Mode of action of warm receptors. *Acta physiol. scand.* 1952 a. *26*. 345—357.

DODT, E. and Y. ZOTTERMAN, The discharge of specific cold fibres at high temperatures (The paradoxical cold). *Acta physiol. scand.* 1952 b. *26*. 358—365.

ENGELMANN, C., Versuche über den Geschmack von Taube, Ente und Huhn. *Z. vergl. Physiol.* 1934. *20*. 626—645.

ENGELMANN, C., Weitere Versuche über den Geschmackssinn des Huhns. *Z. vergl. Physiol.* 1937 a. *24*. 451—462.

ENGELMANN, C., Vom Geschmackssinn des Huhns. *Forsch. Fortschr.* 1937 b. *13*. 425—426.

ENGELMANN, C., Versuche über die „Beliebtheit" einiger Getreidearten beim Huhn. *Z. vergl. Physiol.* 1940. *27*. 525—544.

HENSEL, H. and Y. ZOTTERMAN, The response of the cold receptors to constant cooling. *Acta physiol. scand.* 1950. *22*. 96—105.

HENSEL, H., L. STRÖM and Y. ZOTTERMAN, Electrophysiological measurements of depth of thermoreceptors. *J. Neurophysiol.* 1951. *14*. 423—429.

HENSEL, H. and Y. ZOTTERMAN, The response of mechanoreceptors to thermal stimulation. *J. Physiol.* (Lond.) 1951. *115*. 16—24.

JACOBS, H. L. and M. L. SCOTT, Factors mediating food and liquid intake in chickens. I. Studies on the preference for sucrose or saccharine solutions. *Poultry Sci.* 1957. *36*. 8—15.

KARE, M. R., R. BLACK and E. G. ALLISON, The sense of taste in the fowl. *Poultry Sci.* 1957. *36*. 129—138.

KAUPP, B. F., Anatomy of the Domestic Fowl. W. B. Saunders Co. Philadelphia. 1918.

KING, A. S. and P. M. BIGGS, General anesthesia in G. domesticus for non-survival laboratory experiments. *Poultry Sci.* 1957. *36*. 490—495.

LILJESTRAND, G. and Y. ZOTTERMAN, The water taste in mammals. *Acta physiol. scand.* 1954. *32*. 291—303.

MOORE, C. A. and R. ELLIOTT, Numerical and regional distribution of taste buds on the tongue of the bird. *J. comp. Neurol.* 1946. *84*. 119—131.

OWEN, R., Anatomy of Vertebrates. 1866. *2*. 129—130. Longmans, Green and Co. London.

PFAFFMANN, C., Gustatory nerve impulses in rat, cat and rabbit. *J. Neurophysiol.* 1955. *18*. 429—440.

ZOTTERMANN, Y., The response of the frog's taste fibres to the application of pure water. *Acta physiol. scand.* 1949. *18*. 181—189.

ZOTTERMAN, Y., Action potentials in the glossopharyngeal nerve and in the chorda tympani. *Scand. Arch. Physiol.* 1935. *72*. 73—77.

ZOTTERMAN, Y., Specific action potentials in the lingual nerve of cat. *Scand. Arch. Physiol.* 1936. *75*. 105—119.

ZOTTERMAN, Y., Species differences in water taste. *Acta physiol. scand.* 1956. *37*. 60—70.

Acta physiol. scand. 1959. 46. 152—158.

From the Institutes of Medical Clinic and Medical Pathology of the University of Turin, Turin, Italy and the Institute of Organic Chemistry and Biochemistry, Stockholms Högskola, Stockholm, Sweden.

Studies on the Incorporation of ^{35}S Labelled Cystine into the Leucocytes of Tumour Bearing Rats.

By

BARTOLOMEO BELLION and LUIGI RESEGOTTI.

Received 16 December 1958.

Abstract.

BELLION, B. and L. RESEGOTTI. Studies on the incorporation of ^{35}S labelled cystine into the leucocytes of tumour bearing rats. *Acta physiol. scand.* 1959. *46*. 152—158. — In order to study the influence of the development of a tumour on the leucopoiesis and on the life cycle of leucocytes, the incorporation of intraperitoneally administered ^{35}S labelled cystine into the leucocytes of rats injected with Yoshida ascites sarcoma was investigated. The separation of leucocytes and the preparation of the samples were performed according to the method of WEISBERGER and LEVINE. Sulfur is incorporated in leucocyte proteins of rats injected with ascites tumour at a rate three times as high as in that of normal rats. The loss of ^{35}S atoms from the leucocytes of tumour animals is more rapid, which suggests a shortening of the life span of W.B.C. due to the presence of the tumour. The striking similarity of these results with that obtained by EHRENSTEIN in the case of erythrocytes is emphasized. The possibility that the results obtained are due to the presence of tumour cells in the peripheral blood is discussed and ruled out.

Blood dyscrasias are often observed in the course of malignant neoplasms, even when an invasion of the hemopoietic tissue by the tumour can surely be ruled out. Only in the last years information was obtained, mostly making use of isotopic techniques, on the mechanism of anemia in cancer. The life span of the erythrocytes has been shown to be shortened in patients suffering from various kinds of malignancies (reviewed by HEVESY 1958) at least to a large extent produced by extrinsic factors (BROWN 1950, DEGOWIN, SHEETS and HAMILTON 1950, HYMAN 1954, SHEETS *et al.* 1954, HYMAN, GELLHORN and HARVEY 1956, MILLER *et al.* 1956, DALSANTO 1957). The shortening of the life span of the erythrocytes is not seldom followed by a formation of red corpuscles at an increased rate. A shortening of the life span of the erythrocytes thus does not necessarily lead to anemia. This is conspicuously shown by tumour bearing mice. EHRENSTEIN (1957) found that though the life span of the erythrocytes of such mice was shortened to half of that of controls, their hemoglobin content did not differ. The hyperplasia of the erythropoietic organs in these animals was also exhibited by the several times larger incorporation of ^{14}C into the hemin of erythrocytes of such mice.

The influence of the tumours on the circulating leucocytes and on the leucopoiesis is obscure. The leucocyte count gives very irregular values in the course of a neoplasm: leucocytosis is sometimes found in rapidly growing tumours (WINTROBE 1956 p. 233); a certain relationship can be observed between leucocyte count and site of onset of the neoplasm: for example primary stomach and lung tumours often induce leukemoid reaction (FAHEY 1954, KUGELMEIER 1935, MEYER and ROTTER 1942). No information is at present available on the mechanism of the action exerted by the tumour on leucocytes.

In order to elucidate this problem we studied the incorporation of ^{35}S labelled cystine into the leucocytes of rats inoculated with Yoshida sarcoma cells. Cystine is largely incorporated into the leucocyte proteins. WEISBERGER and LEVINE (1954) observed the appearance of radioactivity in the leucocytes of the peripheral blood as early as 10 min after oral administration of radioactive cystine. The stability of the sulfur atoms of the leucocyte proteins has not been investigated. However, the specific activity curves reported by WEISBERGER and LEVINE are quite similar to those obtained by KLINE and CLIFFTON (1952) by making use of ^{32}P

as a tracer in leucocyte DNA studies. As DNA P is known to have a high stability in the circulating leucocytes (OTTESEN 1954), the leucocyte protein sulfur must be admitted to have a similar stability. The rapid appearance of radioactivity in the leucocytes soon after oral administration of the tracer is possibly due to an additional formation of proteins taking place in the leucocytes after they entered the circulation. A similar phenomenon has been observed by one of the authors (RESEGOTTI 1957), when studying the incorporation of ^{59}Fe into the leucocyte hemins.

Methods.

The experiments have been carried out on 60 male white rats, weighing 100 to 120 g. The animals were fed on a standard diet throughout the experimental time.

The graft of the tumour was performed by injecting i.p. 0.5 ml of ascites fluid secured under sterile condition from animals inoculated with Yoshida's sarcoma. ^{35}S labelled cystine having a specific activity of 9 mC/mM was used as a tracer. Each animal received i.p. a single dosis of 1 μC, 24 hours after inoculation of the ascites.

At different time intervals from the injection of the ^{35}S labelled cystine, the animals were sacrificed by decapitation and the blood was collected in silicone coated tubes with sequestrene as anticoagulant. The separation of leucocytes was obtained by sedimentation of the red corpuscles with dextran. The leucocyte fraction thus obtained is largely contaminated by erythrocytes. These were hemolyzed with 0.16 per cent saponine. The leucocytes were then washed twice with saline. At the end of this procedure a pure suspension of leucocytes, consisting almost completely of lymphocytes was obtained.

The radioactivity of the samples was measured according to the method of WEISBERGER and LEVINE, making use of an end window Geiger-Müller tube, having a 2 mg/cm^2 thick mica window.

From the data obtained the specific activity (counts/min/mg of leucocytes (dry weight)) was calculated.

Results.

Total and differential counts of leucocytes of normal and sarcoma bearing rats are reported in Table I.

The presence of the tumour hardly changed the number of both granulocytes and lymphocytes in the peripheral blood.

The rate of incorporation of radioactive sulfur into the leucocyte with time is shown in Fig. 1.

The specific activity gradually increases until the third day,

Table I.

Total and differential leucocyte counts of normal and tumour rats (mean values).

Days after the graft of the tumour	Total W. B. C.	Granulocytes	Lymphocytes	Tumour cells
2*	10,600	1,160	9,220	no
3*	14,800	1,180	13,300	no
4*	7,500	1,200	6,150	no
5*	13,100	1,700	10,480	no
6*	9,300	840	8,000	no
9*	15,000	2,100	12,500	no
13**.....	12,900	1,800	10,900	***0.5 %
15***.....	14,300	700	13,300	0.8 %
Controls	13,300	1,330	11,500	—

* = 4 animals.
** = 2 animals.
*** = 1 animal.

both in normal and in cancerous animals; in the latter, however, reaching a value three times as high as in the former. The activity then decreases rapidly in the tumour bearing animals and at a minor rate oniy in the normal ones. Thus at the eighth day, such difference in the specific activity of the leucocytes of the two groups of rats is not longer present and the percentage of ^{35}S atoms begins to be higher in the leucocytes of sarcoma bearing animals.

The animals injected with ascites tumour can hardly survive more than 14 days. It was therefore impossible to investigate the change in the radioactivity of the leucocytes of cancerous animals after that date. In the controls the loss of the ^{35}S atoms by the leucocyte proteins takes place after the lapse of 14 days at a very low rate, as shown by Fig. 1.

The specific activity of the tumour cells was determined in 12 rats, sacrificed 2, 3 and 5 days after the injection of ^{35}S labelled cystine. No ascites was found in the peritoneum of the animals killed one day after the administration of the tracer (2 days after the graft of the tumour). In the animals sacrificed after the 8th day the ascites was largely mixed with blood, which might bias the results. In the second day the specific activity of the sarcoma cells (26 counts/min/mg) was higher than that of the circulating leucocytes; but in the third it was already decreased (16 counts/min/mg) in the fifth being only 5 counts/min/mg.

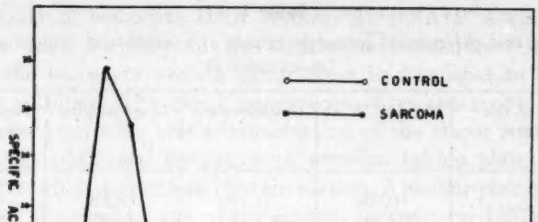


Fig. 1. Rate of incorporation of ^{35}S cystine into the proteins of leucocytes of control and tumour bearing rats. The ordinate is the specific activity of leucocyte protein sulfur. The abscissa is time in days after injection of the tracer.

Discussion.

The method of WEISBERGER and LEVINE has been chosen for the present investigation despite the fact that it does not permit to calculate the life span of leucocytes because it is the most suitable for detecting changes induced by the presence of the tumour in the life cycle of the leucocytes, which is the main interest of our investigation.

The differences in the specific activity curves (Fig. 1) obtained for normal and tumour bearing rats can be explained by an increased rate of both formation and decay of leucocytes in the latter. Our results resemble conspicuously those obtained by EHRENSTEIN. The latter found in tumour bearing mice an enhanced rate of incorporation of ^{14}C into the hemin of erythrocytes due to hyperplasia of the erythropoietic organs and followed by a rapid ^{14}C loss due to the shortened life span of the erythrocytes. No difference was found in the number of erythrocytes present in the circulation of tumour bearing and control mice by EHRENSTEIN. Nor did we find a marked difference in the number of leucocytes.

It has been reported by KELLY *et al.* (1951), PAYNE, KELLY and WHITE (1952) and by MCINDOE and DAVIDSON (1952) that

the presence of a tumour induces an increase of the turnover rate of the DNA even in tissue not directly affected by the tumour. The formation of leucocytes requires among others the formation of DNA. That in the tumour bearing animals DNA formation is accelerated in leucocyte forming organs as well, was shown by the above mentioned authors. The shortening of the life span of the leucocytes in the presence of a tumour may be due to the same lytic factors which attack erythrocytes and the factors responsible for the compensatory increase in the rate of formation of red corpuscles in the tumour bearing organism may be responsible for the increased rate of formation of leucocytes as well.

The higher specific activity of the leucocytes of the sarcoma bearing rats could also be due to the presence in the blood of tumour cells containing a relative high number of ^{35}S atoms. The possibility to find cancer cells in the peripheral blood has been extensively investigated by ENGELL (1955). MOORE, SANDBERD and SCHUBARG (1957) found tumour cells in the blood of 93 patients in a group of 179, having various kinds of cancer. However, their presence can be usually detected only by special techniques as their number is extremely low. More frequent is the finding of tumour cells in the blood of rats bearing Yoshida ascites sarcoma, possibly because of the reticuloendothelial nature of the cells of this tumour. According to YOSHIDA (1949) they can amount to 2.5 per cent of the total number of the blood leucocytes. In the present experiments no tumour cell was found in the blood of almost all the tumour bearing rats. Only in two animals less than 1 per cent of cells which could be considered as tumour cells were observed. As the difference of specific activity between sarcoma cells and leucocytes is rather small, the exceptional presence of very few sarcoma cells in the blood can hardly play a significant role in determining the differences of specific activity observed between normal and tumour bearing rats.

An alternative hypothesis must be mentioned as well. In the man (OTTESEN 1954) and in the rabbit (RESEGOTTI 1957) the leucocyte population has been shown to consist of two groups having a different life span. If this is also the case in the rat it would be conceivable that in the tumour bearing organism the abundance ratio of the short and the long living leucocytes is shifted in favour of the first mentioned one, resulting in an increased specific activity of the leucocytes.

References.

BROWN, G. M., The pathogenesis of secondary anaemias. *Canad. med. Ass. J.* 1950. *62.* 472—475.

DAL SANTO, G., Iron turnover in carcinoma of the uterine cervix. *Acta obstet. gynec. scand.* 1957. *36.* 150—165.

DE GOWIN, E. L., R. F. SHEETS and H. E. HAMILTON, Studies with inagglutinable erythrocyte counts. A method for measurement of net gain or deficit of red cells in the human subject. *J. clin. Invest.* 1950. *29.* 693—699.

EHRENSTEIN, G. v., quoted by HEVESY, G. v., Historical progress of the isotopic methodology and its influence on the biological sciences. *Min. nucleare* 1957. *1.* 182—200.

ENGELL, H. C., Cancer cells in the circulating blood. *Acta chir. scand.* 1955 Suppl. 201.

FAHEY, R. J., Unusual leukocyte responses in primary carcinoma of the lung. *Cancer* (N. Y.) 1954. *4.* 930—935.

HEVESY, G. v., Die Krebsanaemie. *Naturwissenschaften.* 1958. *7.* 247—252.

HYMAN, G. A., Studies on anaemia of disseminated malignant neoplastic diseases. I) The hemolytic factor. *Blood* 1954. *9.* 911—919.

HYMAN, G. A. and J. E. HARVEY, The pathogenesis of anaemia in patients with carcinoma. *Amer. J. Med.* 1955. *19.* 350—356.

HYMAN, G. A., A. GELLHORN and J. E. HARVEY, Studies on the anaemia of disseminated malignant neoplastic diseases. II) Study of the life span of the erythrocyte. *Blood.* 1956. *II.* 618—631.

KELLY, L. S., A. H. PAYNE, M. R. WHITE and H. B. JONES, The effect of neoplasma or pregnancy on the tissue deoxypentose nucleic acids. *Cancer Res.* 1951. *II.* 694—702.

KLINE, D. E. and E. E. CLIFFTON, Life span of leucocytes in man. *J. appl. Physiol.* 1952. *5.* 79—82.

KUGELMEIER, L. M., Leukaemoid Reaktionen bei Carcinom. *Folia haematol.* (Lpz.) 1935. *53.* 370—381.

MCINDOE, W. M. and J. N. DAVIDSON, The phosphorous compounds of the cell nucleus. *Brit. J. Cancer* 1952. *6.* 200—214.

MEYER, L. M. and S. D. ROTTER, Leukemoid reactions (hyperleukocytosis) in malignancy. *Amer. J. clin. Path.* 1942. *12.* 218—222.

MILLER, A., R. B. CHODOS, C. P. EMERSON and J. F. ROSS, Studies of the anaemia and iron metabolism in cancer. *J. clin. Invest.* 1956. *35.* 1218—1262.

MOORE, G. E., A. SANDBERG and J. R. SCHUBARG, Clinical and experimental observations of the occurrence and fate of tumor cells in the blood stream. *Ann. Surg.* 1957. *146.* 580—604.

OTTESEN, J., On the age of human white cells in peripheral blood. *Acta physiol. scand.* 1954. *32.* 75—92.

PAYNE, A. H., L. S. KELLY and M. R. WHITE, Effect of neoplastic tissue on turnover of liver nucleic acids. *Cancer Res.* 1952. *12.* 65—71.

RESEGOTTI, L., Life cycle of granulocytes and lymphocytes of the rabbit determined by making use of ^{59}Fe labelled haemin as a tracer. *Acta physiol. scand.* 1957. *41.* 325—339.

SHEETS, R. F., H. E. HAMILTON, E. L. DEGOWIN and C. D. JANNEY, Studies with inagglutinable erythrocyte count. V) Spontaneous and X-ray induced haemolysis in malignancy. *J. clin. Invest.* 1954. *33.* 179—190.

WEISBERGER, A. S. and B. LEVINE, Incorporation of radioactive L-cystine by normal and leukemic leukocytes in vivo. *Blood* 1954. *9.* 1082—1094.

WINTROBE, M. M., Clinical haematology. VI ed. *Lea and Febiger* N. Y. 1956.

YOSHIDA, T., The Yoshida sarcoma as ascites tumor. *Gann* 1949. *40.* 1—20.

Acta physiol. scand. 1959. *46*. 159—164.

From the Department of Physiology, Kungl. Veterinärhögskolan, Stockholm 51, Sweden.

Inactivation of Insulin in Livers from Mice with Goldthioglucose Obesity.

By

STIG LARSSON.

Received 18 December 1958.

Abstract.

LARSSON, S. Inactivation of insulin in livers from mice with goldthioglucose obesity. *Acta physiol. scand.* 1959. *46*. 159—164. — The insulin inactivating capacity of livers of mice with goldthioglucose obesity has been determined using the method described by MIRSKY and BROH-KAHN (1949). It was found that insulin was destroyed more readily in livers of obese animals compared to normal. It is suggested that these findings can be related to the higher incidence of diabetes mellitus in obesity.

Diabetes mellitus occurs more frequently among people who are over-weight as compared to those whose weights are normal or sub-normal (MARKS 1957). A direct relation between obesity and diabetes mellitus in dogs has also been found (KROOK, LARSSON and ROONEY 1959).

LARSSON (1957) studying obesity caused by a single injection of goldthioglucose found a certain incidence of diabetes mellitus in animals, obese for long periods of time. When rat diaphragms are

incubated with insulin in a glucose medium there are wide variations in the sensitivity of the diaphragm to the hormone (see WILLEBRANDS and GROEN 1954). The present investigation was performed in view of these findings and the series of experiments by MIRSKY (1957) which suggested increased insulinase activity in some types of diabetes mellitus.

Material and Methods.

Male albino mice of the ASw strain were used. The animals were housed in individual cages at a temperature of 24° C. All mice were fed *ad libitum* (mouse crackers, Lindberg, Älvsjö). Once a day the food and water intake and the weights of the animals were measured.

Ten obese and 15 control mice were used for the insulin study. For the water and fat determinations of the liver 6 obese and 15 control animals were used. The obese animals all weighed more than 43.0 g, and the controls not more than 30.5 g. The age of the mice used in the experiments was more than 15 months, the over-weight mice being obese for at least 7 months.

Obesity was produced by a single injection of goldthioglucose (Solganal B-oleosum, Schering). The amount injected to produce obesity was 1 mg per g body weight. This dose was established by BRECHER and WAXLER (1949). The goldthioglucose was injected intraperitoneally in the mice after 36 hours fasting.

None of the animals used had any "clinical" signs of diabetes mellitus. The insulin inactivating capacity of the liver and in some cases of the omental fat was, with some modifications, determined according to the method described by MIRSKY and BROH-KAHN (1949 a). After a regular food intake the animals were killed by decapitation. The livers were immediately removed, weighed and a part taken for histological examination. One gram of the liver was homogenised with 3 g of ice-cold distilled, sterilised water and filtered. After filtration 1 ml of homogenate and 1 ml of an insulin (Vitrum) solution (8 I. U. per ml of sterile saline) were incubated at 35° C for 1 hour. Differences in pH of the homogenates were not taken into consideration. If possible, duplicates were always taken. After incubation, the medium was filtered and injected i. v. into female virgin rabbits which had been fasted for 24 hours. Blood samples for analyses of reducing substances were taken 30 min before the injection, immediately before the injection and then 30 and 60 min after the injection. The blood was analysed for reducing substances according to the Nelson-Somogyi method (SOMOGYI 1952).

Of the other 6 obese and 15 control mice, the water and fat content of the liver were determined as described by FINLAYSON, KROOK and LARSSON (1959). Thus, the liver was dried to determine the water content; afterwards the fat content was determined by extraction with petroleum-ether in a Soxhlet apparatus.

Table I.

Ranges for body and liver weight, water and fat contents of the liver in normal and obese mice.

	Normal	Obese
Body weight ..	24.5 —30.5 (30)	43.1 —84.5 (16)
Liver weight ..	1.00— 1.93 (30)	1.87— 3.14 (16)
Liver water ...	67.5 —74.8 (15)	58.3 —69.9 (6)
Liver fat	7.8 —12.9 (15)	10.9 —27.8 (6)

The weights are expressed in grams; water and fat contents in per cent (the fat content on dry weight basis). The number of animals are given in parentheses.

Results.

Table I shows the body weights, the water and fat contents of the animals used in this study. Because the differences between the normal and fat animals are so large, no statistical analysis but only the limit values are given.

Fig. 1 gives the insulin inactivating capacities of the control and obese mice, expressed as pooled percentages in relation to the pre-injection blood sugar values according to MIRSKY and BROH-KAHN (1949 a).

As can be seen there is a marked difference in the inactivating capacities of normal and obese mice. As almost no inactivation of insulin was observed in the omental fat; these values are not given in the figure.

Histological examination revealed that there was a significantly higher incidence of fatty infiltration of the livers of obese animals compared to the livers of the controls. However, in the "obese" livers only moderate pathological fatty infiltration of the degenerative type was encountered.

Discussion.

The results show that livers from mice with goldthioglucose obesity have an increased insulin inactivating capacity compared with untreated animals of normal weight. Because the increased fat content of the livers of obese animals could be responsible for

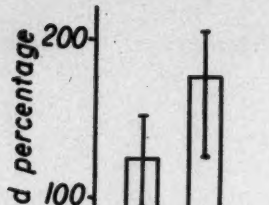


Fig. 1. The insulin inactivating capacities of normal and "obese" livers expressed as pooled percentages according to MIRSKY and BROH-KAHN (1949 a). The vertical lines express the ranges.

this effect, the inactivating capacity of the omental fat was studied. It was found that this fat tissue possessed hardly any inactivating ability at all. The fact remains that the livers of the obese animals have a higher fat content, which was also verified by the histological examination of the liver. The fatty infiltration of the liver of these obese animals was only moderate and no grave pathological lesions of the liver were observed. In a series of experiments MIRSKY *et al.* (see MIRSKY 1957) have studied the nature of the insulin inactivating mechanisms and found evidence that diabetes mellitus in many cases might be caused or exaggerated by an increased insulin destroying capacity of the body, particularly of the liver. In the present study where no diabetes mellitus was found in the animals under investigation, the increased insulin inactivation of the livers of obese animals was similar to that found by MIRSKY in diabetes mellitus. MIRSKY (1957) has discussed the effect of pH of the homogenate on the insulin inactivating properties. In this study, however, pH variations were not taken into consideration, because it was thought that adjusting the pH to a set level would change the true environment of the particular liver sample tested. By and large no great differences existed from sample to sample.

It is known that when rat diaphragms are incubated with insulin in a glucose medium there are wide variations in the sensitivity of the diaphragm to the hormone (see WILLEBRANDS and GROEN 1954). Of the different factors that can influence the response of insulin in such types of studies the following have been mentioned: the mineral composition of the incubation medium; the glucose concentration of the medium; fasting; the age of the animals used and other factors of a more technical character. The present experiments, however, show that even the nutritional condition of the animals is important for the inactivation and thus for the potency of insulin. MIRSKY and BROH-KAHN (1949 b) and MIRSKY, PERISUTTI and DIENGOTT (1957) found a marked reduction of the insulin-inactivating system after fasting. In the present study the mice were killed immediately after a regular food intake. As demonstrated by LARSSON and STRÖM (1957) obese mice have a different feeding pattern from those of normal weight. Thus, it was shown that mice with goldthioglucose obesity eat less frequently but considerably larger quantities every time when compared to the controls. This change in feeding pattern was suggested as a cause of the splanchnomegaly, demonstrated by DRACHMAN and TEPPERMAN (1954). The accommodation to a higher food intake causing increased size and hypertrophy of the abdominal organs also affects the pancreas. It was shown in this study that the larger liver size together with increased insulin inactivating capacity of this organ are also present in the obesity. It has been pointed out in a review by HAAGENSEN (1958) that many obese patients have abnormal, "prediabetic" glucose tolerance curves. In this connection it is of interest to note that many of these patients also have an increased amount of circulating insulin.

The previous findings concerning an increased insulin inactivating capacity in the liver of obese mice suggests an imbalance of the production versus inactivation of insulin. This view is also supported by the higher incidence of diabetes mellitus in human obesity (MARKS 1957), as well as in canine obesity (KROOK *et al.* 1958), and in goldthioglucose obesity (LARSSON 1957).

References.

Brecher, G. and S. H. Waxler, Obesity in albino mice due to single injections of goldthioglucose. *Proc. Soc. exp. Biol.* (*N. Y.*) 1949. *70*. 498—501.

Drachman, R. H. and J. Tepperman, Aurothioglucose obesity in the mouse. *Yale J. Biol. Med.* 1954. *26*. 394—409.

Finlayson, J., L. Krook and S. Larsson, The effect of obesity, pyometra and diabetes mellitus on the fat and cholesterol contents of liver and spleen in the dog. *To be published.* 1959.

Haagensen, N. R., Fedmens pathofysiologi. *Nord. Med.* 1958. *59*. 253—257.

Krook, L., S. Larsson and J. Rooney II., The interrelationship of diabetes mellitus, obesity and pyometra in the dog. *Amer. J. vet. Res.* 1959. In press.

Larsson, S. and L. Ström, Some characteristics of goldthioglucose obesity in the mouse. *Acta physiol. scand.* 1957. *38*. 298—308.

Larsson, S., Food preferences in obesity caused by goldthioglucose. *Acta physiol. scand.* 1957. *40*. 367—376.

Marks, H. H., Relationship of body weight to mortality and morbidity. *Metabolism* 1957. *6*. 417—424.

Mirsky, I. A. and R. H. Broh-Kahn, The inactivation of insulin by tissue extracts. I. The distribution and properties of insulin inactivating extracts (insulinase). *Arch. Biochem.* 1949 *a*. *20*. 1—9.

Mirsky, I. A. and R. H. Broh-Kahn, The inactivation of insulin by tissue extracts. II. The effect of fasting on the insulinase content of rat liver. *Arch. Biochem.* 1949 *b*. *20*. 10—14.

Mirsky, I. A., G. Perisutti and D. Diengott, Effect of fasting on insulinase activity and on hypoglycemic response to insulin. *Endocrinology* 1957. *60*. 303—307.

Mirsky, I. A., Insulinase, insulinase-inhibitors, and diabetes mellitus. *Recent Progr. Hormone Res.* 1957. *13*. 429—471.

Somogyi, M., Notes on sugar determination. *J. biol. Chem.* 1952. *195*. 19—23.

Willebrands, A. F. and J. Groen, The determination of insulin in blood. *Advanc. intern. Med.* 1954. *6*. 331—351.

Acta physiol. scand. 1959. *46*. 165—173.

From the Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm 60, Sweden.

The Discharge to Maintained Stretch of Spindles in Slow and Fast Muscles of Rabbit.

By

RAGNAR GRANIT and SABURO HOMMA.[1]

Received 19 December 1958.

Abstract.

GRANIT, R. and S. HOMMA. The discharge to maintained stretch of spindles in slow and fast muscles of rabbit. *Acta physiol. scand.* 1959. *46*. 165—173. — Systematic measurements of the maintained discharge to stretch at time 4—5 sec from onset have been carried out with 69 muscle spindles in ankle extensors and flexors of anaesthetized rabbits. Three main types of spindles can be separated on the basis of rate of discharge, slow, medium and fast spindles. The number of fast spindles is greater in fast muscle. Slow and medium spindles only are found in slow muscle.

It was noted by GRANIT, SKOGLUND and THESLEFF (1953) when testing muscle spindles with succinylcholine that spindles in tibialis anterior tended to put up higher average discharge rates than those in gastrocnemius or soleus (cat). Recently, when one of us (GRANIT 1958) studied the response of soleus spindles to maintained stretch in the decerebrate cat, frequency of discharge was plotted against extension of the muscle in mm at time 4—5

[1] Present address: Laboratory of Physiology, Chiba University, Chiba, Japan.

sec after onset of pull. There were considerable variations but the average rate of increase for 20 soleus spindles was as low as 3.5 impulses/mm, though the frequencies at zero extension, owing to gamma bias, averaged as much as 26 imp/sec. A limited number (7) of gastrocnemius spindles had been tested in the course of the same experiments. Their slopes averaged out at 6.5 imp/mm. These observations led to the surmise that spindles in slow and fast muscles might differ with respect to their frequency of discharge in maintained stretch.

When for other reasons some experiments were undertaken on rabbits the impression was obtained that in them the range of variation of the slope of the spindles' frequency-extension curves varied much more than in cats. It was then decided to study the response to maintained stretch in slow and fast muslces of this animal. The ankle muscles were used because of the relatively long dorsal roots in which their afferents are represented. In order to compare rabbits and cats measurements of spindle discharge were made at the same time as in the previous work, 4—5 sec after onset of stretch.

Methods.

The animals were given a dose of 5.0 ml/kg of a mixture of 10% urethane and 1% chloralose, one hind leg denervated except for the muscles to be used, laminectomy performed, and the leg fixed at the three joints. Muscle temperature was controlled by a thermocouple. Functionally isolated spindles were identified in thin dorsal root filaments. Conduction time was nearly always taken in order to determine conduction velocity from the nerve electrode at the knee to the dorsal root filament (average conduction distance 15 cm). A minority of the spindles had latent periods between 2 and 4 msec and thus conduction velocities below 75 m/sec, which generally are assumed to be myotube endings (MERTON 1953, HUNT 1954) but most of them had conduction times between 1.2 and 1.5 msec and therefore belonged to the rapidly conducting nuclear bag endings (100—125 m/sec). It was not possible to distinguish slowly conducting spindle afferents from the rapidly conducting ones by features other than conduction velocity.

The muscles used were soleus (S), gastrocnemius (G), plantaris (P), extensor digitorum longus (EDL) and tibialis anterior (TA). Abbreviations within brackets refer to the graph of Fig. 8. The two flexors, TA and EDL, were used with denervated ankle extensors and *vice versa*. All muscles were carefully isolated. Soleus in the rabbit is firmly fixed to the lateral gastrocnemius in the lower part of its belly, but for these experiments was dissected free.

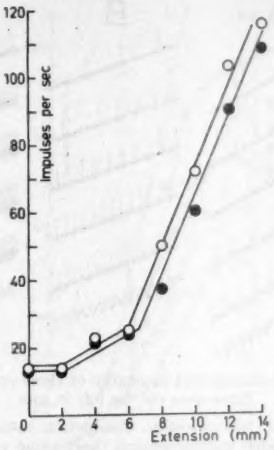


Fig. 1. Maintained discharge of gastrocnemius spindle.

Conduction velocity 100 m/sec.

***Open circles*, before, *filled circles*, after de-efferentation.**

The myograph stand was fixed to a sliding device provided with a catch so that any extension in mm could be reproduced at will. Zero length was determined by the initial length necessary to make the myograph barely record a deflection. This would be a deflection of the order of 20 g.

The preparation just described usually had intact motor innervation of its spindles. This made it sensitive to painful stimuli or twist of the ear but, in between, the spindles discharged with the regular frequency characteristic of absence of, or very low gamma bias. In one animal the spinal cord was destroyed and, occasionally, in others, ventral roots were cut without noticeable influence on the discharge of spindles to stretch. If the basic rhythm of discharge was irregular, suggesting variations in gamma bias, more anaesthesia was given in small doses until the discharge became steady.

However, it is of interest to study the behaviour of a spindle before and after ventral root section in a specific case such as that of Fig. 1. Clearly its response to stretch has not changed after de-efferentation more than one is entitled to expect in comparing two series of stretches repeated at an interval of half an hour. In this time the injury discharge from the cut ventral roots should be gone.

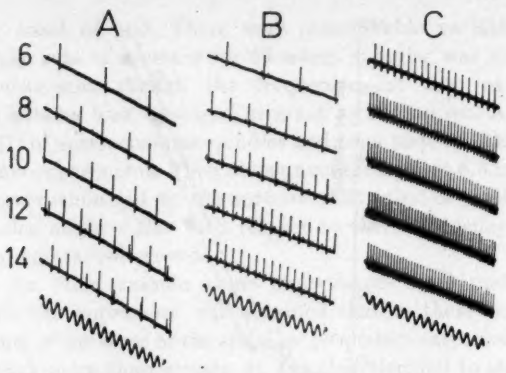


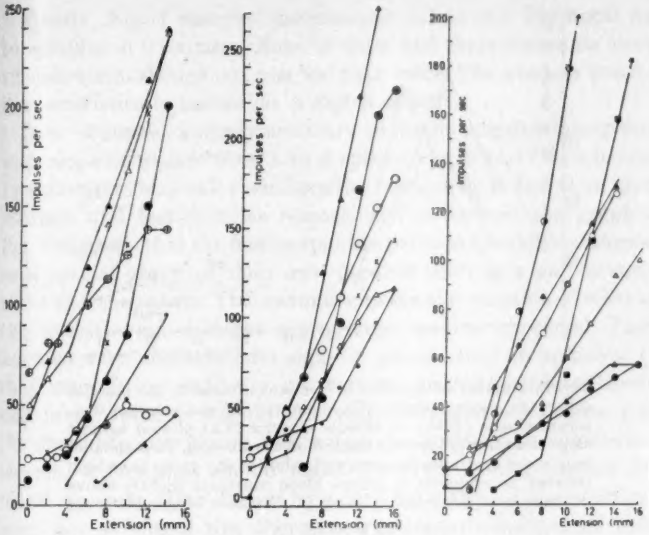
Fig. 2. Samples of maintained discharge of three spindles, A, B and C. Extension on the left in mm.

A: From medial gastrocnemius. Conduction velocity 115 m/sec.
B: From undivided gastrocnemius. Conduction velocity 100 m/sec.
C: From tibialis anterior. Conduction velocity 79 m/sec.
Time, 100 cps.

Results.

With respect to the frequency of discharge at comparable extensions, rabbit spindles may be subdivided into three main types, examples of which in Fig. 2 are shown under A, B and C respectively. The numerals 6 to 14 indicate muscle extension in mm. Rates of discharge are thus slow, medium or fast. Muscle spindles in ankle extensors of cats have been studied for several years in this laboratory, though — with the exception of soleus spindles (GRANIT 1958) — less systematically in relation to extension than in the present work. The general experience of one of us (R. G.) has been that very fast spindles do not occur in cat ankle muscles. However, flexors have but rarely been used for such purposes. Very high frequencies of discharge can, of course, be produced in cats by sufficient gamma bias.

Samples will now be given in Figs. 3—7 of discharge frequencies plotted against extension in mm for spindles in different muscles. Fig. 3 from tibialis anterior (TA) has been chosen to illustrate variations in a single experiment in which 8 spindles were isolated. Clearly this muscle contained spindles of different type but four of them belonged to type C with very fast frequencies of discharge and a slope above 12 imp/mm. The total number of TA spindles



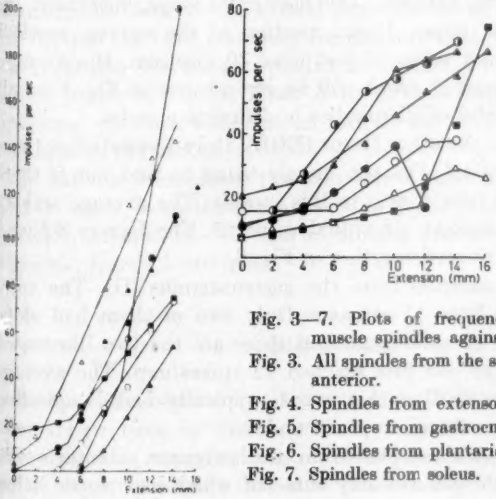


Fig. 3—7. Plots of frequency of discharge of muscle spindles against extension in mm.

Fig. 3. All spindles from the same animal's tibialis anterior.

Fig. 4. Spindles from extensor digitorum longus.

Fig. 5. Spindles from gastrocnemius.

Fig. 6. Spindles from plantaris.

Fig. 7. Spindles from soleus.

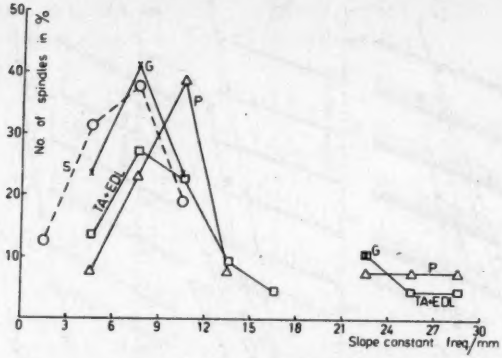


Fig. 8. Number of spindles in per cent of total in the muscles soleus (S), gastrocnemius (G), plantaris (P) and extensor digitorum longus (EDL) + tibialis anterior (TA) plotted against slope constants grouped as marked on the abscissa. Thus spindles with slope constants between end values of any group have been referred to midpoint of group. Slope constants slightly above 30 have been regarded as being 30. Number of spindles is for

$$S = 16, G = 17, P = 13, TA + EDL = 23.$$

was 16, from five animals. The maximum slope, measured, as always, from the upper, linear, portion of the curves, reached the incredibly high value of just over 30 imp/mm, the average being 13 spikes/mm. A graph will be given below in Fig. 8 for all experiments, a total of 69 spindles in different muscles.

Fig. 4 refers to the other flexor (EDL). Only 7 spindles in three animals were studied, the aim merely being to find out if there were spindles of type C also in this muscle. The average was 15 spikes/mm, the highest individual value 25. The flexors TA and EDL have been treated together in Fig. 8.

Fig. 5 shows samples from the gastrocnemius (G). The total was 17 spindles from 11 animals. Only two of them had slope constants above 12 spikes/mm and these are the two illustrated in Fig. 5, another one just reached 12 spikes/mm. The average was 9.8 imp/mm and so this muscle typically had B spindles. However, it also contains types A and C.

Fig. 6 illustrates samples from the extensor, plantaris (P). The total was 13 spindles only three of which had slopes below 8 spikes/mm (5.7, 7.5 and 7.5 respectively). There were four very fast spindles (slopes 12, 24, 26, 30). The average was 13.

Finally, Fig. 7 samples the extensor soleus (S). The total was 16 spindles in 9 animals. None of them had slope constants above 12 spikes/mm though one reached that value. The average was 6.0. Characteristically soleus has A and B spindles.

It is, of course, entirely arbitrary to divide spindles into groups with slope constants from 1 to 8 spikes/mm, 8 to 12 and beyond 12 spikes/mm and call them spindles of type A, B and C, or slow, medium and fast spindles respectively. A look at the graph of Fig. 8 suggests that the fastest spindles perhaps should be separated as a special group of their own because there is a gap between them and the others. This naturally raises the question of whether the fastest spindles might not in some way be abnormal. There are two very characteristic signs of abnormality in spindles: (1) they stop firing suddenly or start to discharge intermittently and many spindles will do this very early under excessive pull, (2) they become phasic and refuse to respond to maintained stretch, a fact that should make the experimenter suspect low blood pressure. Our fastest spindles by either of these criteria were not abnormal. On the contrary, they discharged at these high rates for many seconds beyond the 5 sec needed for the test, even at extensions as high as 12—14 mm. In order not to cause damage one hesitates to extend experiments at great extensions far beyond the time necessary for measurement. One more reason for regarding the fastest spindles as normal is their distribution in the graph of Fig. 8. There is no obvious explanation of why abnormal spindles would be so rare in the slow muscles and so common in the fast ones.

In rabbits contraction times in soleus tend to be between 55 and 60 msec, in gastrocnemius around 27, in plantaris around 20, in EDL around 17—19 and in tibialis anterior which is more variable, from 12—16 msec.

Discussion.

It has been shown by Gordon and Holbourn (1949) that contraction time in muscle twitch varies from fibre to fibre. Many muscles are mixed and in them the slow components tend to lie deeper than the fast ones (Denny-Brown 1929, Gordon and Holbourn 1949, review of earlier literature, Needham 1926), An example is tibialis anterior in the cat which is a fast muscle

(DENNY-BROWN 1929). GORDON and PHILLIPS (1953) dissected out a deep, slow component which in the total twitch merely appeared as an insignificant tail after the fast events. In rabbits such a tail is often seen in the fast EDL. In embryological life intrafusal muscles grow out of extrafusal ones (*e. g.* CUAJUNCO 1927) and so, in the same muscle, may well have different mechanical properties depending upon site. The overall contraction time in twitch merely serves as a general guide. It will, of course, favour the fast components, partly because the slow ones are submerged in the fast twitch but also because slow muscle requires tetani to build up its response.

Now, this research was prompted by the notion that slow extrafusal systems may have correspondingly slow intrafusal ones and that the fast extrafusal fibres in fast muscles might give rise to spindle fibres with similar characteristics. Considering all the facts mentioned above there is nothing in our results that could not be explained by this hypothesis.

On the other hand, there is a very large anatomical literature on spindles from this and the previous century which demonstrates that intrafusal fibres have different lengths (see *e. g.* SHERRINGTON 1894). One may distinguish short and long ones. It is not known how these are distributed in muscles. It is possible that the length of the intrafusal fibre may determine the amount of strain to which the sense organ will be subjected in stretch, provided that the spindle inserts on tendon or aponeurosis as it commonly does. Some spindles, however, insert on perimysium or endomysium at both polar regions. In the absence of precise histological data hypotheses based on intrafusal fibre length and site of insertion are useless. So also are theories based on nuclear bags of different properties. The simple mechanical hypothesis outlined above differs from mere speculation in that it has proved its value. It suggests that excessive pull on soleus should occasionally raise high frequencies of discharge in a spindle. We have seen this happen twice, one case being illustrated in Fig. 7. More often than not, however, soleus spindles stop increasing in frequency at great extensions.

As stated above, the frequency spectrum of spindles from the corresponding muscles in the cat does not extend to the high frequencies seen in the rabbit. What slope constants we possess for soleus and gastrocnemius in the cat also average out at lower values than in the rabbit. Why this should be so we do not know

and can only point to evidence (KERSCHNER 1893, BARKER 1948) according to which the rabbit end organs are less well differentiated into two types, the annulo-spiral and the flower-spray varieties, than are those in the cat. Rabbit end organs are found in the nuclear bag or adjacent myotube regions of the spindles but tend to be rather alike in appearance (BARKER 1948). Of the two fanciful names, the 'flower spray' fits rabbit spindle organs better. Conduction velocity is no definite guide. The afferent fibre of one of the extremely fast spindles depicted in Fig. 4 (slope constant 25) in EDL had a conduction velocity of only 60 m/sec but then, in soleus, there were a number of equally slowly conducting spindle afferents. No obvious correlation could be detected between conduction velocity and slope constant. In gastrocnemius the average conduction velocity was 112 m/sec, in EDL + TA only 75 m/sec, estimated from the conduction time on the basis of an average distance of 15 cm.

This work has been supported by the Swedish Medical Research Council.

References.

BARKER, D., The innervation of the muscle spindles. *Quart. J. micr. Sci.* 1948. *89*. 143—186.

CUAJUNCO, F., Embryology of the neuromuscular spindle. *Carnegie Inst. Contrib. Embryol.* 1927. *19*. 45—93.

DENNY-BROWN, D. E., The histological features of striped muscle in relation to its functional activity. *Proc. Roy. Soc. B.* 1929. *104*. 371—411.

GORDON, G. and A. H. S. HOLBOURN, The mechanical activity of single motor units in reflex contractions of skeletal muscle. *J. Physiol.* (*Lond.*) 1949. *110*. 26—35.

GORDON, G. and C. G. PHILLIPS, Slow and rapid components in a flexor muscle. *Quart. J. exp. Physiol.* 1953. *38*. 35—45.

GRANIT, R., Neuromuscular interaction in postural tone of the cat's isometric soleus muscle. *J. Physiol.* (*Lond.*) 1958. *143*. 387—402.

GRANIT, R., S. SKOGLUND and S. THESLEFF, Activation of muscle spindles by succinylcholine and decamethonium. The effects of curare. *Acta physiol. scand.* 1953. *28*. 134—151.

HUNT, C. C., Relation of function to diameter in afferent fibers of muscle nerves. *J. gen. Pnysiol.* 1954. *38*. 117—131.

KERSCHNER, L., Über die Fortschritte in der Erkenntnis der Muskelspindeln. *Anat. Anz.* 1893. *8*. 449—458.

MERTON, P. A., Slowly conducting muscle spindle afferents. *Acta physiol. scand.* 1953. *29*. 87—88.

NEEDHAM, D. M., Red and white muscle. *Physiol. Rev.* 1926. *6*. 1—27.

SHERRINGTON, C. S., On the anatomical constitution of nerves to skeletal muscles; with remarks on recurrent fibres in the ventral spinal nerve-root. *J. Physiol.* (*Lond.*) 1894. *17*. 211—258.

Acta physiol. scand. 1959. *46*. 174—184.

From the Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm 60, Sweden.

Phasic Stretch and 'Spindle Constant' in Slow and Fast Rabbit Muscle.

By

RAGNAR GRANIT and SABURO HOMMA.[1]

Received 19 December 1958.

Abstract.

GRANIT, R. and S. HOMMA. Phasic stretch and 'spindle constant' in slow and fast rabbit muscle. *Acta physiol. scand.* 1959. *46*. 174—184. — The aim of this paper is to find a mathematical expression for the inherent properties of muscle spindles as indicators of phasic changes. Rate of change of muscle extension and rate of change of interval between two successive spindle discharges have been measured and plotted, one against the other. Their ratio has been found constant at different rates of stretch. This is the spindle constant, defined in the paper.

It is well known from the work of MATTHEWS (1933) that the muscle spindle responds to rate of stretch as well as to maintained pull of the muscle. In the afferent terminals of individual muscle spindles in the frog KATZ (1950) recorded equivalent dynamic and static changes of 'generator potential'. It is hardly necessary to quote further papers in support of what is common knowledge,

[1] Present address: Laboratory of Physiology, Chiba University, Chiba, Japan.

namely that fast stretch of limited amplitude may raise higher discharge frequencies than slow pull extending the muscle to considerably greater length.

The discharge frequency in maintained stretch varies a great deal from spindle to spindle but the relation between frequency and extension is linear (ELDRED, GRANIT and MERTON 1953, GRANIT 1958). We have recently (GRANIT and HOMMA 1959) found this rule well obeyed with rabbit muscle spindles and have also shown that there is a general relation between contraction time of a muscle and the slope of the frequency/extension-curve of its spindles. The slope tends to be small in slow muscle and steep in fast muscle. Nothing is known, however, of the phasic properties of different types of spindles in relation to rate of extension. This paper attempts to fill this gap in our knowledge.

The biological background of this work is partly an interest in receptor properties as such but mainly a desire to understand principles of rapid control in the particular situation when a brief contraction of one muscle stretches its antagonist at the same joint, and the muscles concerned — as often is the case — have different contraction times.

With this in mind we have developed a technique of stretching, the principle of which is that one ipsi- and one contralateral ankle muscle are jointed to the opposite ends of a horizontal lever pivoting around its midpoint. With this arrangement, when one muscle is made to contract by a tetanus to its nerve, pull will be exerted on the contralateral fellow muscle at the opposite end of the lever. Thus rates of stretch of a physiological order of magnitude can be conveniently obtained and it is possible to measure rate of extension together with the discharge frequency of an indicator spindle from the extended muscle. It is necessary in such work to use soleus for graded contractions because fast muscles such as the ankle flexors accelerate the spindle too rapidly for accurate measurements of frequency.

Methods.

Rabbits were anaesthetized by an intravenous injection of a mixture of 1 % chloralose and 10 % urethane (5 ml/kg). Ether was given during the operation but not during the experiments. This is the preparation already described in the previous paper (GRANIT and HOMMA 1959), with the difference only that the animals always were de-efferented

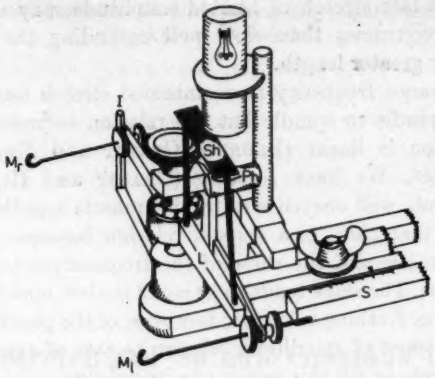


Fig. 1. Diagram of apparatus, as described in text.

from L5 to the end of the sacral roots. Spindle afferents were isolated in the dorsal roots, as described. Muscles were isolated and the leg denervated as described in the previous paper.

The essential new feature of the experiment is the apparatus shown in Fig. 1. *L* is the horizontal lever mentioned in the introduction. It moves on ball bearings. Right and left ankle extensors (M_r and M_l) are attached to it by threaded hooks. By altering the length of these hooks, the correct initial alignment of the lever, as shown by the indicator *I*, is achieved. Light illuminates a photocell (*Ph*) whose exposed end faces a shutter (*Sh*) attached to the left lever arm. By this arrangement, the output of the photocell is directly proportional to movement of the lever arm, and hence, to change in muscle length. The strain gauge on the same arm records tension. The stand holding the midpoint of the lever can be shifted along the scale (*S*), so as to set initial length. The amplified outputs of photocell and strain gauge are led to one double beam cathode ray tube to give records of extension and tension respectively (see figures).

The impulses discharged by the indicator spindle are led to a pair of amplifiers in parallel. The output of one is displayed as a conventional record of spikes; the other amplifier drives an interval recorder, used in several papers from this laboratory and designed by Dr. Bernhard Frankenhaeuser. The output of the interval recorder is shown on the other beam of the same tube. In a typical interval record (Fig. 3 b) the height of each vertical stroke is directly proportional to the interval between two successive impulses and hence, inversely proportional to frequency. Joining the peaks of a series of these strokes gives the curve of change of impulse intervals. Direct recording of spindle discharge is merely a running control of the interval recorder, because the latter is sensitive to spike amplitude and may fail if this diminishes while a measurement is in progress.

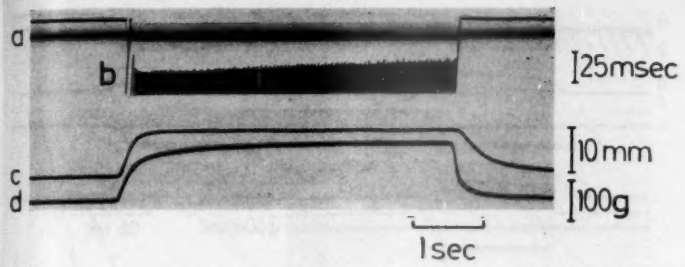


Fig. 2. Soleus stretched by contraction of contralateral soleus (tetanization of tibial nerve).

a: Spindle discharge from dorsal rootlet of L7.
b: Spindle discharge is led to interval recorder; the vertical height of each stroke is proportional to interval between two successive spikes. Calibration of 25 msec interval.
c: Extension curve recorded by photocell. Calibration of 10 mm.
d: Tension curve. Calibration of 100 g.

In the actual experiments muscle M_r was the right soleus which is tetanized from its muscle nerve. The lever then pulls on the left muscle M_l which in our experiments has been soleus, plantaris or, occasionally, tibialis anterior. In this way different smooth rates of exponential stretch can be obtained by varying the frequency of the supramaximal tetanic stimulus.

Results.

In Fig. 2 the two lower curves (*c* and *d*) show extension and tension respectively, in one de-efferented soleus when the other is tetanized for 5 sec. The upper records illustrate spindle discharge in the opposite soleus directly (*a*) and by the interval recorder (*b*). Tension (which in all records is the thicker line) as well as directly recorded discharge frequency (*a*) merely serve as general controls. All measurements to be presented below deal with mm extension (*c*) and interval (*b*) in msec which is easier to measure than its inverse value, the discharge frequency. It has also turned out that interval lends itself more readily to quantitative treatment than frequency. In Fig. 2 adaptation is well shown by the slowly rising contour of the peaks of the interval record. This figure is shown to illustrate the full course of the events at slow film speed.

For measurements the film has been run at higher speed, as

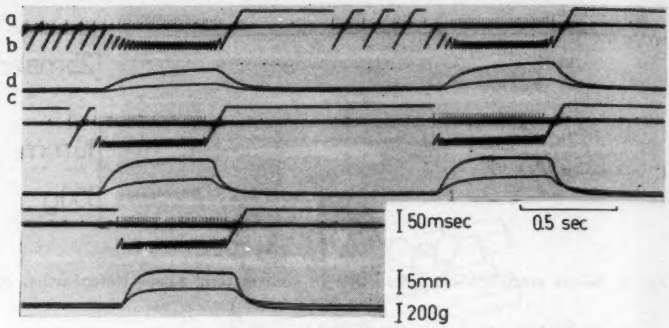


Fig. 3. As Fig. 2, but five experiments at increasing rates of extension from 1 to 5. Extension 3.4 mm. Note calibrations of interval recorder, strain gauge and lever excursion measuring extension.

in Fig. 3. We shall be concerned solely with the time from zero to maximum extension or minimum interval, respectively. Fig. 3 shows successive records of stretch of soleus at different rates of extension. Otherwise it is similar to Fig. 2. These records will now be analyzed in detail.

Extension: Let E_t be the extension at any early time t from zero onwards, and E_f the final semistationary state of extension which, from the point of view of the present problem, may be regarded as a stationary maximum.

Measured points on the five curves of Fig. 3 are plotted in Fig. 4 a on semilogarithmic paper. It is seen that log $(E_t - E_f)$ plots as a straight line against t, but only during the first 200 msec. However, this is the time during which discharge intervals rapidly shorten to reach a final minimum and, so, is the time in which we are interested. The lines drawn are described by the simple exponential equation

$$E_t = E_f(1 - e^{-t/\beta}), \qquad (1)$$

in which β is the slope constant given by the numerals opposite each line of Fig. 4 a. It will be called the extension constant and its dimensions are time. For the present purpose equation 1 is a good enough approximation to our data.

The extension constant β decreases from 400 at slow rates of pull to 55 with fast extension.

Discharge intervals: Let I_f be the final minimum interval (in

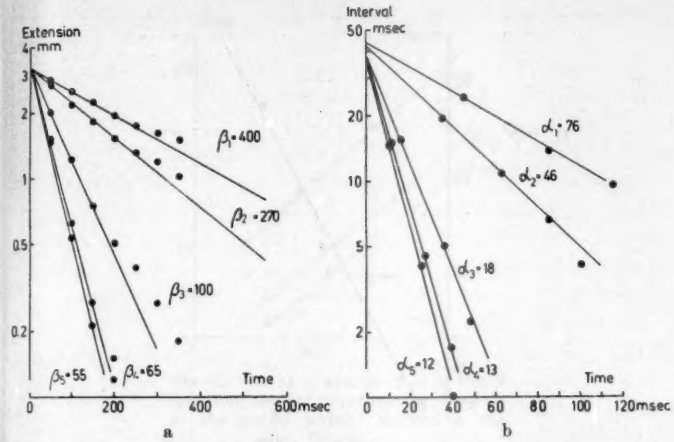


Fig. 4. Analysis of Fig. 3. Logarithmic ordinates. From above downwards the five curves refer to the experiments 1—5 of Fig. 3.

a: Relation between time and extension, latter defined as $E_f - E_t$. β_1, $\beta_2 \ldots \beta_5$ are the extension constants calculated from equation 1 (see text).

b: Relation between time and interval, defined as $I_t - I_f$. Curves drawn according to equation 2. α_1, $\alpha_2 \ldots \alpha_5$ are the interval constants (see text).

each of the five experiments of Fig. 3), I_t the interval at any early time t, and I_i the initial interval. These were found to be related by the equation

$$I_t = I_f + I_i\, e^{-t/\alpha}. \qquad (2)$$

The points measured in the experiment are plotted in Fig. 4 b. There is a good fit when log $(I_t - I_f)$ is plotted against t. The slope of each line is an interval constant α, expressed in msec. It is seen that the values of α, just as those of β, decrease with faster pull.

With these data available it is possible to relate rate of extension to rate of change of interval. Fig. 5 shows the interval constant α plotted against the extension constant β. Clearly their ratio is constant and for this particular spindle β/α is 5.0. This ratio serves to characterize the sensory organ in phasic stretch and will be called the *spindle constant.*

Significance of spindle constant. Having shown how the spindle constant is obtained the next step is to demonstrate the biological value of this concept. The first comparison concerns slow and

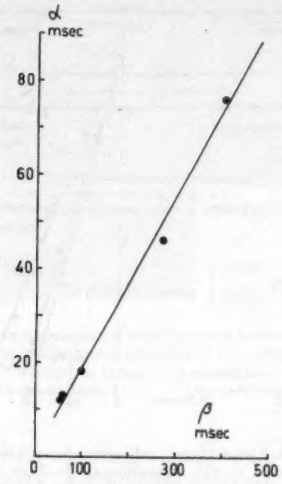


Fig. 5. Demonstration of constant ratio between interval constants α and extension constants β of Fig. 4. The spindle constant is defined as the ratio β/α which in the present case is 5.0.

fast muscle. In the rabbit (GRANIT and HOMMA 1959) soleus is slow, plantaris has a large fast component but also a small, slow one, tibialis anterior is fast and does not seem to possess the well-defined slow component that GORDON and PHILLIPS (1953) found in the cat's tibialis. However, it is likely that most ankle muscles contain fibres of different contraction times. The dominant type will dominate the myographic record. A number of spindle constants measured in the manner demonstrated are shown in

Table 1.

Soleus	Plantaris	Tibialis anterior
7.1	5.0	2.7
5.2	3.2	2.1
5.2	3.1	—
5.0	2.7	—
5.0	2.1	—

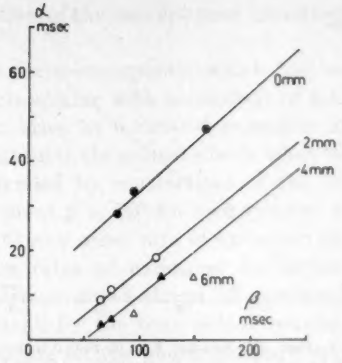


Fig. 6. Plot of α against β as in Fig. 5 but from another experiment with soleus at the initial lengths marked in the figures.

Thus, in the rabbit, the spindle constants are large in slow muscle and small in fast muscle. Values for tibialis anterior were difficult to obtain for reasons stated above. The spindle constants thus serve to differentiate spindle types in slow and fast muscle from the point of view of their phasic responses. In the case of tonic (steady) stretch, when spindle frequency is plotted against extension, a linear graph is obtained whose slope for a spindle in slow muscle is less than one in fast muscle, demonstrating a similar differentiation in spindle responses (GRANIT and HOMMA 1959).

Very generally, large spindle constants mean that fast rates of extension do not play the same role for the discharge of the sense organ as they do when the spindle constant is small. In the latter cases a little change in rate of extension greatly influences discharge rate. Slow muscles containing spindles with large spindle constants, when pulled upon at different rates by their antagonists, therefore will not signal variations in rate of stretch as efficiently as fast muscles in which the spindle constants are small.

The question next arises as to whether different levels of tonic gamma activity might influence the spindle constants. To a first approximation gamma activity is likely to cause a steady contraction of the muscular poles of the spindle and so be equiv-

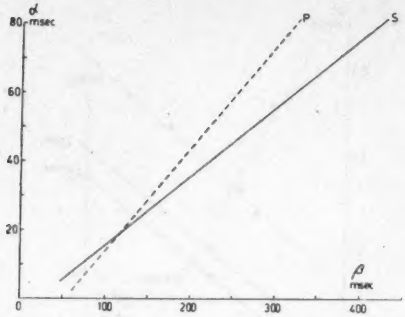


Fig. 7. Plot of α against β as in Fig. 5 in experiment in which one soleus spindle (S) and one plantaris spindle (P) were analyzed in succession, both muscles being at the same initial length.

alent to an 'internal' variation in length of the nuclear bag. This effect can be imitated by starting stretch at different initial lengths.

Fig. 6 is an experiment with a soleus muscle at 0, 2, 4 and 6 mm initial length. It is seen that the three curves for 0, 2 and 4 mm are parallel when α is plotted against β, as in Fig. 5. (Only two values could be obtained at 6 mm owing to the technical difficulties mentioned, when frequencies of discharge are high.) Thus the spindle constant as such does not depend upon the extent to which the nuclear bag is biased by internal stretch caused by tonic gamma activity contracting the muscular poles. It is an inherent property of the sensory organ. GRANIT and HOMMA (1959) came to the same conclusion with regard to the variations of the slope constants in static stretch, mentioned above.

Inspection of Fig. 6 shows that the interval constant a diminishes under stretch (or gamma activity causing intrafusal stretch). This means that good gamma bias or an equivalent amount of extension makes the spindle more sensitive to fast phasic changes, as experimentally found by GRANIT and HENATSCH (1956).

Fig. 5 has shown that, for a given spindle, the rate at which the interval changes is proportional to the rate at which extension changes. If, in one and the same experiment, one plantaris- and one soleus-spindle are selected and the same initial length is used for both muscles, it is possible to visualize directly an im-

portant implication of the concept here introduced by the 'spindle constant'.

S in Fig. 7 is the soleus spindle which had a constant of 5.0, *P* is the plantaris spindle with a constant of 3.1. The two curves in α—β diagram cross at a rate of extension around 120 msec. Assume the situation in the animal's body when these two synergist muscles are extended by contractions of the flexor antagonists. At the crossing point $\beta = 120$ the two spindles will give the same information α. At any other rate of extension than $\beta = 120$ they will differentiate rates of extension by differential behaviour. When rate of stretch grows slower (β increases), α continues to be reasonably small for the tonic soleus spindle but undergoes a considerable increase for the phasic plantaris spindle. This means that the soleus spindle, owing to its large spindle constant, senses slow phasic changes better than the plantaris spindle. Again, when rate of stretch increases to the left of the crossing point (β decreases); the faster plantaris spindle, owing to its small spindle constant, becomes the relatively better instrument of the two, because α grows small for it and changes very little for the soleus spindle.

In this experiment the crossing point was actually measured by the two curves of Fig. 7. This finding suggests that intrafusal contraction in a set of spindles may, by altering spindle length, displace their α—β curves along the α-axis (without altering their slopes) and thereby shift the crossing point in an appropriate direction — yet another example of what incredibly fine instruments for control the muscle possesses in its spindles. Intrafusal contractions are monitored both by reflexes and by supraspinal events (see summary, GRANIT 1955). Hitherto we have mostly considered tonic aspects of intrafusal activity. Considerations based on the spindle constant seem to offer a valuable line of approach to an understanding of integrations based on phasic differentiation.

Discussion.

Time is hardly ripe for a discussion of applications of the concept introduced here because no experimental work exists which compares rates of extension in a group of co-operating muscles. One does not know how the reflex effects emerge in terms of tonic and phasic motoneurones and muscles, still less is

it possible, on the sensory side, to go beyond the general statements made above that the new findings show the existence of grid of β/a curves which will shift their relative position and points of intersection under the influence of stretch and/or intrafusal control. Information from the muscle is bound to vary accordingly.

Summary.

1. Exponential stretch of tonic and phasic muscles in rabbits has been carried out by a special device (Fig. 1). Rate of change of extension of muscle (β) and rate of change of interval (a) between individual discharges of an indicator spindle has been measured.

2. It is shown that for any one spindle β/a is a constant, large in tonic muscle and small in phasic muscle. This is defined as the spindle constant.

3. The spindle constant is independent of initial muscle length.

4. Considerations based on plots of spindle constants show that spindles in fast muscles respond better to fast rates of extension, spindles in slow muscles better to slow rates of extension.

The authors are indebted to the Swedish Medical Research Council for a grant in aid of this work.

References.

ELDRED, E., R. GRANIT and P. A. MERTON, Supraspinal control of the muscle spindles and its significance. *J. Physiol.* (*Lond.*) 1953. *122*. 498—523.

GORDON, G. and C. G. PHILLIPS, Slow and rapid components in a flexor muscle. *Quart J. exp. Physiol.* 1953. *38*. 35—45.

GRANIT, R., Receptors and Sensory Perception. *New Haven, Yale University Press* 1955. pp. 369.

GRANIT, R., Neuromuscular interaction in postural tone of the cat's isometric soleus muscle. *J. Physiol.* (*Lond.*) 1958. *143*. 387—402.

GRANIT, R. and H.-D. HENATSCH, Gamma control of dynamic properties of muscle spindles. *J. Neurophysiol.* 1956. *19*. 356—366.

GRANIT, R. and S. HOMMA, The discharge to maintained stretch of spindles in slow and fast muscles of rabbit. *Acta physiol. scand.* 1959. 165—173.

KATZ, B., Depolarization of sensory terminals and the initiation of impulses in the muscle spindle. *J. Physiol.* (*Lond.*) 1950. *111*. 261—282.

MATTHEWS, B. H. C., Nerve endings in mammalian muscle. *J. Physiol.* (*Lond.*) 1933. *78*. 1—53.

Acta physiol. scand. 1959. *46*. 185—193.

From the Nobel Institute for Neurophysiology,
Karolinska Institutet, Stockholm 60, Sweden.

Prolonged Changes in the Discharge of Mammalian Muscle Spindles Following Tendon Taps or Muscle Twitches.

By

RAGNAR GRANIT, SABURO HOMMA[1]
and PETER B. C. MATTHEWS.[2]

Received 25 October 1958.

Abstract.

GRANIT, R., S. HOMMA and P. B. C. MATTHEWS. Prolonged changes in the discharge of mammalian muscle spindles following tendon taps or muscle twitches. *Acta physiol. scand.* 1959. *46*. 185—193. — The muscle spindle is a very sensitive recording instrument and is here found to change its level of excitability, despite de-efferentation, after tendon taps or submaximal contraction, as shown by prolonged changes in its frequency of discharge. It is suggested that this effect often is due to mechanical displacement, but since it is influenced by injection of Flaxedil, the intrafusal end plates may well be spontaneously active in the normal state. Flaxedil causes full paralysis of extrafusal motor end plates at a time when intrafusal gamma end plates are far less influenced.

[1] Present address: Laboratory of Physiology, Chiba University, Chiba, Japan.
[2] Present address: Laboratory of Physiology, Oxford University, Oxford, England.

It is well known that the release of a stretched muscle is often followed by a period of silence in the discharge of its muscle spindles (MATTHEWS 1933). We were therefore surprised when, in the course of other work, we found that a brief tap to the tendon of a muscle might sometimes initiate a prolonged increase in the discharge of the muscle spindles from it. This paper describes investigations into the phenomenon, unfortunately without coming to any definite decision as to its origin. A major difficulty has been that the effect has sometimes been more easily elicitable with tendon taps, sometimes with twitches below the strength necessary for stimulating gamma motoneurones. However, some factors have been experimentally excluded.

Methods.

The results to be described have been obtained from muscle spindles lying in the ankle extensor muscles of both cats and rabbits. The cats were anaesthetized with ether and decerebrated by suction, usually just in front of the anterior colliculi. In some cases they were then made spinal by cutting the spinal cord at the level of Th12. The experiments were performed when the effect of the ether had worn off. Some rabbits were similarly decerebrated and made spinal under ether anaesthesia. Other rabbits were anaesthetized by the intravenous injection of a mixture of 1 % chloralose and 10 % urethane (5—6 ml/kg), and not decerebrated. A little ether was also usually given during the operation but not during the experiment. The anaesthetized rabbits had little if any tonic gamma activity but gamma reflexes to pinna manipulation and to painful stimuli could be elicited.

Single muscle spindle afferents were isolated by splitting dorsal rootlets into fine filaments and recording from their peripheral ends. Fuller details have been given in many preceding publications from this laboratory (*cf.* GRANIT and KAADA 1952, ELDRED, GRANIT and MERTON 1953, GRANIT 1958). The leg studied was firmly fixed, and the nerves other than those to the extensor muscles were cut. Sometimes the whole triceps surae was used, but mostly the gastrocnemius and soleus muscles were separated. In the rabbit the gastrocnemius is firmly attached to a portion of the soleus in the lower half of it. Separation therefore entails some mutilation of either muscle. The experiments were usually performed under both isometric and isotonic conditions for recording the response of the muscle. For isometric recording the muscle was connected by metal links to a strain gauge myograph on a movable stand. For 'isotonic' recording one of these metal links was replaced by a light spring (stiffness either 40 g/mm or 9 g/mm), thus allowing the responses of the muscle spindle to be followed after the muscle had shortened.

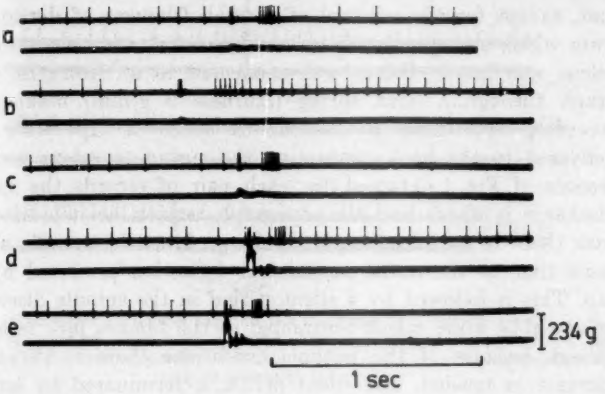


Fig. 1. The effect of tendon taps on the discharge of a muscle spindle in the soleus muscle of a decerebrate cat (pre-collicular). For each pair of records: above, the discharge of the muscle spindle recorded from a small filament of dorsal root; below, myograph record obtained from an isometric myograph connected to the soleus muscle by a weak spring (stiffness 9 g/mm). The records are arranged in order of increasing size of tap. Note that the discharge after the tap is in some cases above the resting level, and in some cases below.

Spindle afferents were identified as such by the pause in their discharge during the contraction of the muscle produced by stimulating the muscle nerve electrically. In rabbits the conduction velocity of the afferent fibre studied was always roughly estimated by determining the time interval between a shock to the muscle nerve and the arrival of the action potential of the single afferent in the dorsal rootlet. The conduction distance was about 15 cm and the conduction times ranged from 1.2 to 3 msec, giving conduction velocities ranging from 125 to 50 m/sec. Some of the slower afferents presumably originated in the myotube endings, but in our tests there have been no obvious differences between the behaviour of the larger and the smaller spindle afferents, and both appeared to be influenced by the gamma motoneurones (*cf.* HUNT 1954). In cats previous experience has shown that the technique of root splitting almost always isolates fibres of high conduction velocity, and we have only occasionally measured it in the present experiments.

Results.

1. *General description.* The prolonged effect which a tendon tap may have on the discharge of a muscle spindle is shown in Fig. 1. The spindle lay in the soleus muscle of a decerebrate cat,

and, except for the removal of a small filament of dorsal root from which the spindle afferent was isolated, the innervation of soleus was intact. Soleus was connected to an isometric myograph through a light spring (stiffness 9 g/mm) making the recording approximately isotonic. A series of taps were then delivered to the hook connecting the spring to soleus and the records of Fig. 1 obtained (for each pair of records the spindle discharge is above and the myograph tension below). In every case there is an initial rapid discharge from the spindle at the same time as the initial mechanical deflection produced by the tap. This is followed by a silent period in the spindle discharge, presumably while soleus contracted in the tendon jerk reflex — though because of the isotonic conditions there is very little increase in tension. The silent period is terminated by another rapid discharge. These are classical findings; what is perhaps surprising is the variation in the discharge of the spindle later in the records, where sometimes the frequency of discharge is above the initial level and sometimes below. There appeared to be no correlation with the strength of the tendon tap, and the records are arranged in order of increasing strength of tap as judged by the mechanical record (the taps were delivered by hand). In this particular experiment the excitatory effect was no longer found when the spring was removed and the soleus connected to the myograph isometrically instead of isotonically; instead the spindle discharge was uninfluenced by the tap. In other experiments the increase in discharge has been found under isometric conditions and this is shown in Fig. 2 a. Here, after two tendon taps, the discharge has increased, above the initial level. In general, however, the prolonged increase in discharge has been more easily obtained under isotonic recording conditions. With isometric registration demonstration of the effect often required careful adjustment of muscle length. It was seen at one length (tension) but not at another. Some stretch was necessary. The effect has been found with afferents conducting at 50—70 m/sec, presumably coming from the myotube endings, as well as with larger afferents from the nuclear bag endings.

A prolonged decrease in the discharge has been particularly marked when the previous discharge rate has been high, especially if it has been raised either by stimulating the muscle nerve at about the strength required to excite the gamma fibres, or if the gamma motoneurones have been excited by a pinna reflex or by

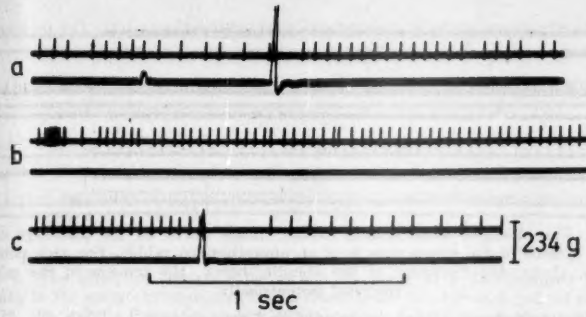


Fig. 2. The effect of tendon taps on the discharge of a muscle spindle in the triceps surae of an anaesthetized rabbit. For each pair of records: above, the discharge of the muscle spindle; below, the tension in the muscle recorded directly with an isometric myograph.

In *a*: the second of two tendon taps increases the discharge.
In *b*: the brain stem was stimulated electrically thereby increasing the frequency of discharge of the spindle.
In *c*: a further tendon tap now reduces the discharge to its original level (spikes retouched).

stimulating in the mid brain. This last effect is illustrated in Fig. 2 b where the effect of mid brain stimulation is shown and in Fig. 2 c where is shown the effect of a subsequently applied tendon tap. This may be compared with Fig. 2 a where is shown the previously excitatory effect of a tendon tap.

In all these cases the stimulus to the muscle spindle system is complex. Initially the tap stretches the muscle, but the ensuing reflex contraction, when present, shortens it again. (With 'isotonic' recording the muscle may shorten to below its initial length, when it will be re-extended by the spring as it relaxes.) In either isometric or isotonic recording the muscle, and its included muscle spindles, will then return gradually to their previous length (this may involve internal redistribution of displacement). Possibly the final level of muscle spindle discharge will depend upon whether the muscle reaches its initial length again by being pulled out, or by shortening. A further complicating factor in the interpretation of the records is that in the intact animal the gamma motoneurones might be affected reflexly either by the initial stretch of the muscle or by its subsequent contraction. However, we shall proceed to demonstrate (section 3) that all the essential features of the prolonged discharge are seen in animals devoid of all reflexes.

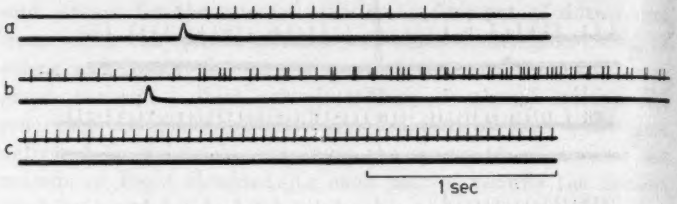


Fig. 3. The effect of weak stimuli to the muscle nerve on the discharge of a muscle spindle in the soleus muscle of an anaesthetized rabbit. For each pair of records: above, the discharge of the spindle; below, the tension in the soleus recorded isometrically.

In *a*: the third shock started the spindle discharging;
in *b*: is shown the ninth shock;
in *c*: is the steady discharge built up after many seconds stimulation (spikes retouched).

2. *Contraction.* The effect of the contraction was investigated by stimulating the nerve to the muscle electrically with weak shocks, submaximal for the contraction of the muscle and therefore considered to be below threshold for the gamma fibres and probably also of the smaller afferent fibres. The shock could also sometimes be set so as to be below the threshold of the spindle afferent studied, but presumably some other spindle afferents or Golgi tendon organ afferents were excited as well as some of the motor fibres. Such an experiment is shown in Fig. 3 taken under isometric conditions. The spindle was initially silent, after the third shock it began to discharge (shown in *a*), the sixth shock is shown in *b*, and in *c* is shown the semistationary discharge established after several seconds of stimulation. Often only one shock was needed but otherwise these were fairly typical results and at that stage suggested that the prolonged excitatory effect of the tendon tap may be partly dependent upon the muscle contraction rather than directly on the initial tap. As some afferent fibres were also excited electrically it is possible that this (reflexly) was the cause of the alteration in spindle discharge. This possibility was next tested by de-efferentation.

3. *De-efferentation.* The effect of a reflex through the gamma motoneurones was initially investigated by giving Flaxedil intravenously in order to paralyse neuromuscular transmission and produce a reversible de-efferentation. In general the prolonged excitatory effects were less marked after this though they could still occur. However by this method it proved impossible to

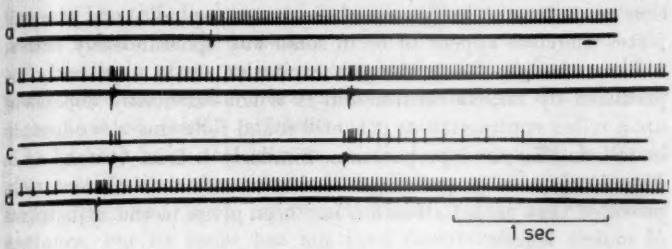


Fig. 4. The effect of tendon taps on the discharge of a de-efferented muscle spindle in the gastrocnemius muscle of a decerebrate cat. For each pair of records: above, the spindle discharge; below, myograph record from isometric myograph connected to the gastrocnemius via a spring of stiffness of 40 g/mm.

a, b: show the increase in discharge produced by tendon taps;
c: is after an intravenous injection of Flaxedil (6 mg), discharge decreased;
d: after extending gastrocnemius by 2 mm when the effect of tendon tap was restored (spikes retouched).

produce a complete gamma block at the time of complete alpha block. Interestingly the gamma end plates were far more resistant to this blocking agent than the alpha end plates on extrafusal muscle, as has also independently been found by HENATSCH and SCHULTE (1958) in the frog. Complete de-efferentation was next employed. In some cases after this the excitatory effect could no longer be obtained, yet in most cases it was still strongly present provided that the length of the muscle was correctly set. An example is shown in Fig. 4, where the recording is again under isotonic conditions. Here *a* and *b* show the effect of three tendon taps and again illustrate the variability of the reactions (note, however, that tendon taps were delivered manually). It seemed of interest to try blocking the end plates in this case also, so Flaxedil was then given, with the result that the resting discharge and the response to tendon tap decreased — Fig. 4 c. Stretching soleus by 2 mm restored both to near their previous level — Fig. 4 d. Further injections of Flaxedil produced the same effect at the increased length, and again the discharge could be restored by stretching. This effect of Flaxedil was regularly found in the completely de-efferented preparation and so cannot be ascribed to the progressive deterioration of the preparation. Also it was seen half an hour after de-efferentation when it seems unlikely that any injury discharges would be persisting. Thus partial paralysis of the end plates in the completely denervated prepara-

tion can alter the behaviour of the muscle spindle, and the end plates therefore appear to be in some way spontaneously active.

Thus though the prolonged excitation may apparently be produced by muscle contraction, it is not essentially dependent upon reflex contraction as it is still found following a tendon tap in the de-efferented preparation. Similarly a brief twitch, as in Fig. 3, may elicit prolonged excitation after de-efferentation, provided that proper attention had been given to the adjustment of muscle length.

Discussion.

In pursuing these experiments it had been hoped to uncover a proprioceptive reflex from contracting muscle to its gamma motoneurones. Though this may occur the present experiments provide little evidence for it, but they do serve to emphasize the lability of muscle spindles after mechanical disturbance. A possible explanation for these results is that after a mechanical displacement the part of the muscle spindle lying beneath the sensory ending does not return to precisely the same length as before. This would make our effect analogous to the prolonged increase in discharge sometimes found by KUFFLER, HUNT and QUILLIAM (1951) after stimulation of the gamma motor fibres. However, it is not quite clear on this view why more basic stretch is required after Flaxedil to elicit the same phenomenon and why it had a lower threshold before the intrafusal end plates were partially paralyzed. The essential effect may therefore be a variation in the spontaneous rate of discharge of gamma motor end plates. The alpha motor end plates have been found to discharge miniature potentials at a faster rate when stretched (FATT and KATZ 1952, HUTTER and TRAUTWEIN 1956). The sensory portion of the muscle spindle may well be a sensitive detector of such spontaneous variations in its motor end plates, some of which, besides, on anatomical evidence (see *e. g.* BARKER 1948) belong to fibres of the alpha group.

Summary.

1. When a tap is given to the tendon of a leg extensor muscle it may be followed by a prolonged (many seconds) increase or

decrease in the discharge in a single afferent coming from a muscle spindle in the muscle.

2. Similarly after one or several brief twitches from a shock to the large muscle efferents a prolonged increase of the discharge of an isolated spindle afferent may ensue.

3. In both cases this increase in discharge may still be found after cutting the ventral roots. This effect is not therefore necessarily dependent upon reflex activation of the gamma motoneurones, but its cause has not been discovered.

This work has been supported by the Swedish Medical Research Council.

References.

BARKER, D., The innervation of the muscle spindles. *Quart. J. micr. Sci.* 1948. *89*. 143—186.

ELDRED, E., R. GRANIT and P. A. MERTON, Supraspinal control of the muscle spindles and its significance. *J. Physiol.* (*Lond.*) 1953. *122*. 498—523.

FATT, P. and B. KATZ, Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* (*Lond.*) 1952. *117*. 109—128.

GRANIT, R., Neuromuscular interaction in isometric postural tone of cat soleus muscle. 1958. *In course of publication.*

GRANIT, R. and B. R. KAADA, Influence of stimulation of central nervous structures on muscle spindles in cat. *Acta physiol. scand.* 1952. *27*. 130—160.

HENATSCH, H.-D. and F. J. SCHULTE, Einflüsse von Curare und Flaxedil auf die Muskelspindeln des Frosches. *Arch. exp. Path. Pharmak.* 1958. *234*. 247—263.

HUNT, C. C., Relation of function to diameter in afferent fibers of muscle nerves. *J. gen. Physiol.* 1954. *38*. 117—131.

HUTTER, O. F. and W. TRAUTWEIN, Neuromuscular facilitation by stretch of motor nerve endings. *J. Physiol.* (*Lond.*) 1956. *133*. 610—625.

KUFFLER, S. W., C. C. HUNT and J. P. QUILLIAM, Function of medullated small-nerve fibers in mammalian ventral roots: Efferent muscle spindle innervation. *J. Neurophysiol.* 1951. *14*. 29—54.

MATTHEWS, B. H. C., Nerve endings in mammalian muscle. *J. Physiol.* (*Lond.*) 1933. *78*. 1—53.

Acta physiol. scand. 1959. *46*. 194—200.

From the University Institute for Experimental Medical Research, Ullevaal Hospital, Oslo, Norway.

Peripheral Circulatory Response to Submersion Asphyxia in the Duck.

By

KJELL JOHANSEN and JOHN KROG.

Received 26 December 1958.

Abstract.

JOHANSEN, K. and J. KROG. Peripheral circulatory response to submersion asphyxia in the duck. *Acta physiol. scand.* 1959. *46*. 194—200. — The peripheral circulation in the duck during diving has been studied. Changes in peripheral blood flow were indicated by measuring the venous pressure-rise in a branch of the femoral vein, when the main venous return was occluded. Electrocardiograms and arterial pressures were recorded simultaneously. Upon diving of the animals an extreme bradycardia was observed while the arterial pressure underwent only small changes. The slow drop in the arterial diastolic pressure during diving indicated a great reduction in the rate of emptying from the larger arteries. The peripheral blood flow determined from the degree of venous return was likewise found to be markedly reduced. It is concluded that diving induces an adjustatory shut down of the circulation through the whole limb. This explains the animals' ability to compensate for the extreme bradycardia in the maintenance of high systemic blood pressure, thus securing an ample circulation to the brain during asphyxia.

The physiological mechanisms responsible for the high tolerance to asphyxia in diving mammals and birds, have so far not been found qualitatively different from those existing in typical land animals. The study of the adjustments to asphyxia are, therefore, most naturally sought for in the diving animals.

The present study deals with the peripheral vascular control in the duck during diving asphyxia.

The idea that asphyxia causes a redistribution of the circulation in such a way that the blood-flow through the brain is increased while peripheral blood-flow is reduced, has been the object of several previous investigations. LENNOX and GIBBS (1932) demonstrated that increased carbon dioxide in the blood of man, was followed by such a circulatory rearrangement. In 1937 IRVING demonstrated that inflation of the lung of the beaver and muskrat was followed by a similar circulatory redistribution. The same phenomenon was likewise described for typical land mammals like cats, dogs and rabbits (IRVING 1938). IRVING further demonstrated that clamping of the trachea elicited the same vascular changes.

In the present study the problem is approached by measuring the degree of venous return from the extremities of ducks during asphyctic conditions.

Methods.

Domestic ducks (*Anas boscas*) were used as experimental animals in the present investigation. The animals were fastened to a specially built board, which could easily be tilted into a bath. A slight cutaneous, local anaesthesia was applied in the femoral region, where an incision was made and the femoral vessels dissected free. A catheter for pressure recording was inserted into a branch of the femoral vein. A ligature was placed around the main femoral vein proximal to the inserted catheter. By means of this ligature the venous return from the extremity could easily be occluded and released at will. Arterial pressures were recorded in the femoral artery or in a branch of this vessel. Electrocardiograms and pressures were recorded simultaneously on a Sanborn recorder.

Results.

Pressure relations in the vencus system.

The venous pressures recorded at normal conditions varied from 15—20 mm Hg and showed small respiratory variations.

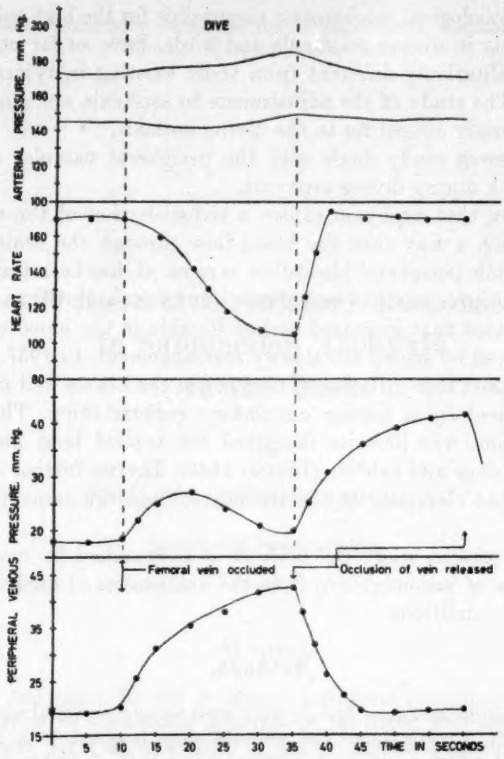


Fig. 1. The curves show from above systolic and diastolic arterial pressure, heart rate and venous pressure in the extremity of the duck before, during and after a dive with venous stasis as marked by the arrows.

The lowest curve was recorded at normal breathing conditions and expresses variations in venous pressure upon occlusion and release of the femoral vein.

By occluding the main femoral vein the pressure rapidly rose to around 40—45 mm Hg in about 25 sec and then levelled off at this value. Upon release of the occlusion the pressure fell rapidly to 15—20 mm Hg in about 10 sec. A representative curve showing these relations is presented at the bottom of Fig. 1.

When the main venous return was obstructed simultaneously with diving of the animals, as seen in Fig. 1, second curve from below, the pressure-rise was only slight and transient. The highest

values, around 25 mm Hg, were attained after approximately 10 sec, after which the pressure slowly fell to near prediving values, like those with the vein unobstructed. If now the animals were emerged, maintaining the venous stasis, a rise in venous pressure almost similar to that presented in the lower curve of Fig. 1, was recorded.

These results seem to bring strong, direct evidence that the peripheral blood flow during diving asphyxia is markedly decreased.

Heart rate.

The third curve from below in Fig. 1 demonstrates the heart rate before, during and after a dive.

The wellknown bradycardia associated with diving was easily detected. In our experiments the reduction in heart rate amounted to around 50 % in about 25 sec. The prediving rate was very rapidly re-established following a dive.

Pressure in the femoral artery.

The arterial pressure undergoes only slight changes at the moment of diving. Thus the mean pressure in the femoral artery was not found to change appreciably in spite of the steadily developing bradycardia. Most often a slight, transient decrease in the diastolic pressure occurred, while the systolic pressure often increased slightly in the first 30 sec of a dive. The pressure in the femoral artery during a short dive (25 sec) is demonstrated at the top of Fig. 1. Following prolonged diving, however, and especially after rediving of the animals, the mean arterial pressure was somewhat lowered. The decrease, however, was rarely more than 25 % of the initial value. Fig. 2 demonstrates the pressure relations in a prolonged dive (3 min).

The extraordinary gentle slope of the diastolic part of the pressure curve in Fig. 2 points toward a considerable retardation of the rate of emptying from the larger arteries, thus compensating for the induced diving bradycardia.

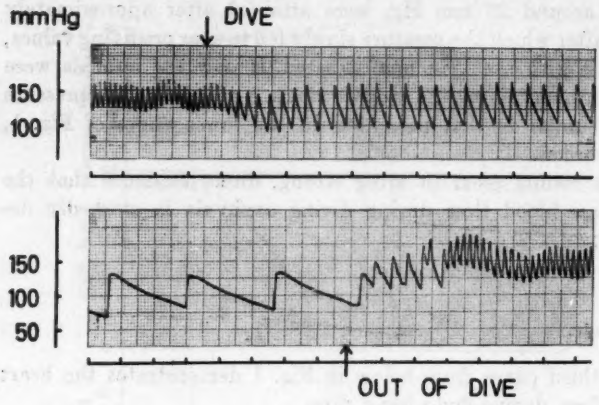


Fig. 2. Tracings demonstrating arterial pressures during a prolonged dive (3 min).

Upper tracing: Entrance to dive.

Lower tracing: Coming out of dive 3 min later.
Time, seconds.

Discussion.

Much effort has been paid to verify the theory that mammals possess the ability to adjust their circulation so as to secure an ample supply of arterial blood to the brain during asphyctic conditions.

Evidence in favour of this theory has, however, not been too well founded experimentally. The confusing results depend to a great extent upon inadequate methods for the measurement of blood flow. Thus the earliest reports utilized arterio-venous oxygen difference or direct observation of the smaller vessels as an indication for changes in blood flow. Great progress was made when IRVING (1937) conceived the idea that natural divers, *e. g.* muskrats, beavers, seals, most reasonably possess the adjustatory mechanisms in an accentuated form. His flow measurements were based on the "thermostromuhr" principle.

Later, GRINELL, SCHOLANDER and IRVING (1942) have extended their investigations and brought further evidence to the problem from experiments on seals. In one experiment (IRVING, SCHOLANDER and GRINELL 1942) the arterial pressure difference

between the femoral artery and a toe artery was used as an indication of changes in peripheral blood flow.

The present study on the duck confirms the results obtained by IRVING *et al.* It may be objected that variations in volume capacity of the occluded extremity may mask blood flow changes through the muscles. However, during normal breathing conditions such volume changes was found to be of minor importance. During diving the initial pressure-rise soon stopped and then fell slowly towards prediving pressure (Fig. 1, second curve from below). This fall might depend on a change in the venous distensibility under these conditions.

The steep pressure-rise immediately after the dive in Fig. 1 (second curve from below) is almost identical with the pressure-rise following occlusion of the femoral vein during normal breathing conditions (Fig. 1, bottom curve). It seems therefore beyond doubt that the blood flow through the extremities of the duck is markedly reduced during diving.

The venous pressure during diving with the venous return unobstructed fell some 10 mm Hg. This, however, gives no proof for a reduced venous return as long as the same situation would result from changes in myocardial distensibility and degree of diastolic filling.

The suggestions of PAPPENHEIMER (1941) experimenting on dogs, that there exists an intramuscular bypass, preventing the blood from contact with the metabolizing parts of the muscles, do not seem probable in the present experiments. Likewise, shunting of blood through arterio-venous anastomoses, as proposed by SCHOLANDER (1940), does not seem likely in our experiments.

The present study indicates that an adjustment to diving seems to be a general reduction of the circulation through the whole limb by contraction of the arterioles. This seems the most natural explanation for the animals' ability to maintain the systemic blood pressure in the presence of the extreme bradycardia during diving.

References.

GRINELL, S. W., L. IRVING, P. F. SCHOLANDER, Experiments on the relation between blood flow and heart rate in the diving seal. *J. cell. comp. Physiol.* 1942. *19*. 341.

IRVING, L., The respiration of the beaver. *J. cell. comp. Physiol.* 1937. *9*. 437.

IRVING, L., Changes in the blood flow through the brain and muscles during the arrest of breathing. *Amer. J. Physiol.* 1938. *122*. 207.

IRVING, L., P. F. SCHOLANDER, S. W. GRINELL, The regulation of arterial blood pressure in the seal during diving. *Amer. J. Physiol.* 1942. *135*. 557.

LENNOX, W. G. and E. L. GIBBS, The blood flow in the brain and the leg of man and the changes induced by alteration of blood gases. *J. clin. Invest.* 1932. *11*. 1155.

PAPPENHEIMER, J. R., Vasoconstrictor nerves and oxygen consumption in the isolated, perfused hindlimb muscles of the dog. *J. Physiol.* (*Lond.*) 1941. *99*. 182.

SCHOLANDER, P. F., Experimental investigations on the respiratory function in diving mammals and birds. *Hvaalraadets skrifter* 1940. No. 22. 131. *Det Norske Videnskaps-akademi i Oslo.*

Acta physiol. scand. 1959. *46*. 201—208.

From the Department of Physiology, Karolinska Institutet, Stockholm 60, Sweden.

Effect of Reduced Ventilation on Systemic Blood Pressure and Blood Flow in the Hind Part of the Cat during Infusion of Noradrenaline.

By

H. DUNÉR and U. S. v. EULER.

Received 9 January 1959.

Abstract.

DUNÉR, H. and U. S. v. EULER. Effect of reduced ventilation on systemic blood pressure and blood flow in the hind part of the cat during infusion of noradrenaline. *Acta physiol. scand.* 1959. *46*. 201—208. — The effect of infusion of 1.6—2.2 μg/kg/min noradrenaline (NA) on the blood pressure and the blood flow in the hind part of the cat in nembutal anaesthesia was studied during normal and reduced ventilation. During normal ventilation NA infusion caused an increase both in blood pressure and flow while both effects were greatly diminished during reduced ventilation. It was further observed that in the latter condition the blood pressure response to carotid occlusion was still strong, in contrast to the effect of NA on systemic pressure.

While the effect of noradrenaline on circulation in certain conditions of shock and circulatory failure is striking and manifests itself in increased systemic blood pressure and improvement of blood flow, it has also been observed that infusion of noradrenaline sometimes exerts an insignificant action only. Since the conditions under which noradrenaline — like the normal vasoconstrictor

outflow — acts on the circulatory system is of importance not only for the understanding of the circulatory homeostasis in pathophysiological situations but also has therapeutical implications, some experiments have been performed in the cat with a view to obtain some information on these points. In previous experiments (DUNÉR and EULER 1957) some factors influencing the secondary fall in blood pressure after infusion of noradrenaline in the cat were studied. The experiments to be reported here are concerned with the influence of reduced ventilation on the blood pressure response and the blood flow during and after infusion of noradrenaline in the cat.

Methods.

Cats anaesthetized with nembutal 35 mg/kg i. p., were used. The arterial blood pressure was measured by a Hg manometer from the right carotid artery. Infusions of noradrenaline, usually 10 μg/ml in saline, acidified with HCl to pH 4, were given by a slow infusion apparatus through a polythene tube inserted in one femoral vein. Infusions were given during 5 or 10 min periods in doses of 1.6—2.2 μg/kg/min.

Moderate degrees of asphyxia were produced by adjusting the artificial respiration after exclusion of spontaneous respiratory movements by a sufficient dose of curare or decamethonium (C10). The muscle paralysants were given repeatedly when necessary.

The reactivity of the cardiovascular system was tested at intervals by occlusion of the left carotid. In some experiments one or both vagi were cut in the neck.

The blood flow of the hind part of the cat was measured by means of an electromagnetic flow meter (SHIPLEY and WILSON 1951) which was connected to the abdominal part of the aorta a few cm above the bifurcation through a polythene cannula of approximately the same width as the aorta. The aortic blood flow was allowed to return to the peripheral part of the aorta. The flow was recorded either by reading off a galvanometer or by continuous recording on a Speedomax recorder.

Results.

Effects on blood pressure by intravenous infusion of noradrenaline during spontaneous and varying degrees of artificial ventilation.

In the dose range used, 1.6—2.2 μg/kg/min, infusion of noradrenaline generally caused a rapid rise in arterial blood pressure. In some experiments the blood pressure remained increased at a fairly constant level. In other experiments, however, the blood

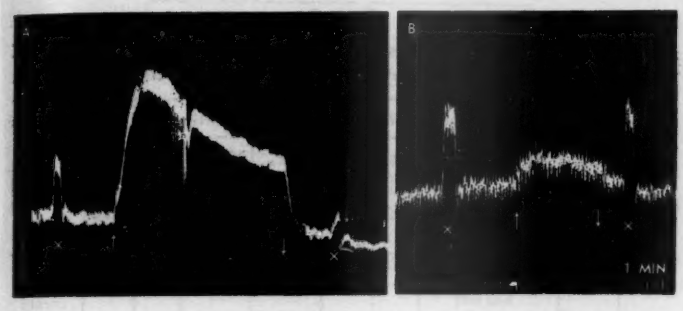


Fig. 1. Blood pressure, cat, nembutal.

A: Spontaneous respiration. Between arrows i. v. infusion of noradrenaline during 10 min at a rate of 1.9 μg/kg/min.

B: Suboptimal artificial ventilation. Animal received 1 + 1 mg decamethonium. Between arrows noradrenaline infusion as in A for 5 min. At × carotid occlusion. Ordinate, 120—300 mm Hg.

pressure, after having reached a peak in about 2 min, gradually fell off as described in a previous paper (DUNÉR and EULER 1957). After the end of the infusion a more or less conspicuous secondary fall occurred on many occasions. The fall was sometimes considerable and the original blood pressure level was often not reached before ½ hour. Repeated infusions as a rule had similar effects.

During adequate artificial respiration after the administration of curare or decamethonium the blood pressure was maintained at the same level as during spontaneous respiration. The blood pressure response to infused noradrenaline then was the same as during spontaneous respiration. By reducing the stroke of the respiration pump a moderate degree of asphyxia was produced as shown by an immediate rise in blood pressure. The effect of a noradrenaline infusion on the blood pressure now was very markedly changed as seen in Fig. 1. Instead of the usual brisk rise in blood pressure which is more or less well maintained, the rise in pressure occurred slowly and was much smaller or even absent. After the end of the infusion no secondary fall occurred in these experiments.

Table I shows the response to infusion of NA during normal and reduced ventilation in 6 experiments.

In a previous report (DUNÉR and EULER 1957) it was noted that the blood pressure response to carotid occlusion was regularly

Table I.

Effect of NA infusion on the systemic blood pressure and the carotid occlusion response during normal and reduced ventilation in the cat.

Exp. no.	NA Dose µg/kg per min	Normal ventilation				Reduced ventilation				Remarks
		B. P. bef. inf. mm Hg	Max. rise in B.P. during NA inf.	Car. occl. resp.		B. P. bef. inf. mm Hg	Max. rise in B. P. during NA inf.	Car. occl. resp.		
				Bef.	After			Bef.	After	
1	2.1	125	82	—	—	135	22	—	—	85 mg curare
2	2.2	156	72	50	35	212	32	80	50	Vagot. curare 70 mg
3	1.7	140	52	76	88	165	18	110	112	Vagot. curare 57 mg
4	1.9	200	45	—	—	235	15	—	—	1 mg C10
5	1.7	135	85	[1]44	[1]16	182	23	66	66	2 mg C10
6	1.6	152	92	[1]14	[1]13	187	28	35	27	1 mg C10

[1] before curare or C10.

diminished for a period of 5—20 min after the end of an infusion of NA in doses of 1 µg/kg/min and upwards. This effect was consistently noted also in the present series of experiments during adequate ventilation (Fig. 1). It was striking, however, that during reduced ventilation after administration of decamethonium or curare, the carotid occlusion test was as a rule increased and only moderately diminished after an infusion of NA which in itself had very much less action (Fig. 1).

Effect of NA infusion on blood flow in the hind part of the cat during normal and reduced ventilation.

When blood flow was measured in the hind part during NA infusion a marked increase was observed as long as ventilation was adequate (Fig. 2). During reduced ventilation, when the blood pressure response to NA infusion was strongly depressed, no change in flow was observed, however (Fig. 3). If ventilation was

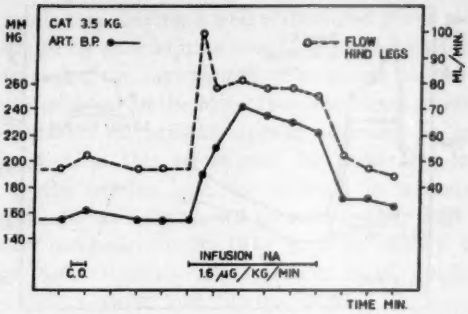


Fig. 2. Cat, nembutal. Effect of NA infusion 1.6 µg/kg/min on the blood pressure and the blood flow through the hind part during normal ventilation. C. O. carotid occlusion. Time min.

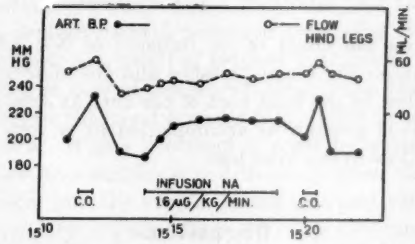


Fig. 3. Cat, nembutal. Effect of NA infusion 1.6 µg/kg/min on blood flow and blood pressure during reduced ventilation. C. O. carotid occlusion.

again increased, the blood pressure response to NA became larger, although usually not as big as the original response. The flow in the hind legs also showed a definite increase. In some experiments a considerable fall in blood flow was noticed when NA was infused during reduced ventilation, particularly at the end of an experiment and in animals showing a marked secondary fall in blood pressure (Fig. 4).

Blood flow in the hind part of the cat during secondary fall in blood pressure after noradrenaline infusion.

In order to obtain some information about the circulatory changes accompanying the secondary fall in blood pressure following an infusion of NA the blood flow in the hind part was measured also during this period.

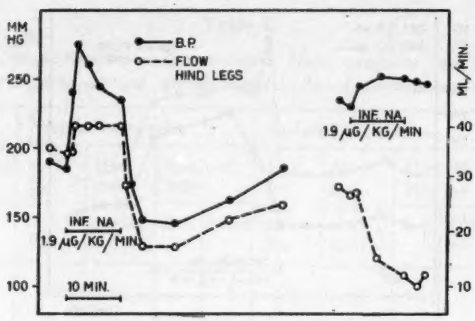


Fig. 4. Cat, nembutal. Effect of NA infusion 1.9 µg/kg/min on blood flow and blood pressure. Marked secondary fall in blood pressure and blood flow. To the right: Effect of NA infusion during reduced ventilation.

Fig. 4 shows the effect of an infusion of NA 1.9 µg/kg/min during 10 min on the blood pressure and the concomitant variations in the flow of the hind part of the cat. As seen in the figure the fall in blood pressure is accompanied by a very marked fall in the blood flow in the hind legs.

Discussion.

The present series of experiments have shown that the secondary fall in blood pressure after an infusion of NA of about 2 µg/kg/min (DUNÉR and EULER 1957) is accompanied by a marked fall in the blood flow through the hind part. It is possible that this effect is mainly due to vasodilatation with pooling of the blood on the venous side and decrease of the circulatory blood volume, but an inhibitory effect on the heart cannot be ruled out. Since it occurs also after bilateral vagotomy, a central or reflex effect mediated via the vagus nerves can be excluded. It is of interest to note that even moderate doses of NA cause a marked effect of this kind. Thus as long as the infused NA acts directly on the vascular wall, the blood pressure is increased and usually well maintained, while the inhibitory effect soon after the end of the infusion results in a long-lasting depression. It should be noted, however, that after moderate doses of NA the inhibitory effect is not a constant phenomenon although it often occurs during the prevailing experimental conditions. At any rate it may occur in animals which seem

to be in a good state, having a well maintained blood pressure and what appears to be an adequate spontaneous respiration.

The reasons for the conspicuous alterations in the effect of noradrenaline infusion on the blood flow and blood pressure during reduced ventilation at present appear obscure. It seems possible, however, that this effect may be partly due to acidosis, secondary to the oxygen lack, in addition to accumulation of carbon dioxide. It has been shown previously that dogs subjected to respiratory acidosis (30 % CO_2) were refractory to pressor drugs (PAGE and OLMSTED 1951, HOULE *et al.* 1957) (cf. also MCDOWALL 1957, GÖMÖRI and TAKÁCS 1956).

The striking discrepancy between the diminished blood pressure response to NA infusion during inadequate artificial ventilation and the very marked blood pressure response to carotid occlusion merits special consideration. Fig. 1 illustrates that the response to NA infusion and to carotid occlusion thus may be changed in opposite directions as a result of reduced ventilation. This is somewhat surprising in view of the general assumption that the carotid occlusion effect on the blood pressure is mediated by the adrenergic neurotransmitter. If this is correct it must be assumed that the locally released transmitter acts differently from the circulating NA on the heart and the vessels during this particular condition, or that the vascular regions affected by the two kinds of stimuli are different. Another possibility would be that the outflow from the suprarenal glands is greatly increased when the carotids are occluded during reduced ventilation.

Whatever the cause of this apparent discrepancy between the effects of the endogenous transmitter and exogenous noradrenaline, which was noted both at high and low systemic blood pressure levels, the diminished effect of NA must undoubtedly be of considerable practical importance when NA is given therapeutically during conditions of inadequate ventilation. Whether or not tissue hypoxia and hypercarbia, as a consequence of inadequate blood flow, may lead to the same failure of NA to exert its full effect on the circulatory system remains to be shown. It appears probable, however, judging from the experience of NA infusion therapy in some conditions of circulatory failure.

If the results obtained in the present study can be applied to similar conditions in man, the practical implication will be that NA infusion as a means of raising an inadequate blood pressure, or improving an unsatisfactory blood flow, is less likely to be

effective during states of hypoxia or hypercarbia. On the other hand the effect of NA should be favourable as long as the respiratory gas tensions in tissues and blood are normal.

Summary.

1. The blood flow through the hind part of the cat is greatly diminished during the secondary fall in blood pressure following infusions of NA of 1.6—2.2 μg/kg/min.

2. During adequate ventilation infusion of NA is accompanied by a considerable rise in blood flow through the hind part of the cat.

3. The rise in blood pressure during infusion of NA is strongly diminished during reduced artificial ventilation after curare or decamethonium.

4. No change or a decrease in blood flow was observed during NA infusion during reduced ventilation.

5. The rise in blood pressure due to carotid occlusion may be markedly increased during reduced ventilation although the response to NA infusion is greatly diminished.

6. Some practical implications for the therapeutic use of NA as a means of improving blood pressure and circulation are discussed.

This work has been supported by a grant from the State Research Committee on Aviation and Naval Medicine. We wish to thank Mr. N.-Å. Persson for skilled assistance.

References.

Dunér, H. and U. S. von Euler, Secondary fall in blood pressure following noradrenaline infusion in the cat. *Acta physiol. scand.* 1957. *38*. 355—363.

Gömöri, P. and L. Takács, Kreislaufregulation bei Hypoxie. *Z. ärzl. Fortbild.* 1956. *7/8*. 286—295.

Houle, D. B., M. H. Weil, E. B. Brown Jr. and G. S. Campbell, Influence of respiratory acidosis on ECG and pressor responses to epinephrine, norepinephrine and metaraminol. *Proc. Soc. exp. Biol.* (N. Y.) 1957. *94*. 561—564.

McDowall, R. J. S., The effects of lack of oxygen on the circulation. In "Control of the circulation of the blood". *Dawson* London. 1957. 238—250.

Page, I. H. and F. Olmsted, The influence of respiratory gas mixtures on arterial pressure and vascular reactivity in "normal" and hypertensive dogs. *Circulation* (*N. Y.*) 1951. *3*. 801—819.

Shipley, R. E. and C. Wilson, An improved recording rotameter. *Proc. Soc. exp. Biol.* (*N. Y.*) 1951. *78*. 724—728.

Acta physiol. scand. 1959. *46*. 209—227.

From the Pharmacological Institute, University of Uppsala, Sweden.

The Binding of Zinc to Glycerol-extracted Muscle, and Its Relaxing Effect.

By

K. A. PAUL EDMAN.

Received 10 January 1959.

Abstract.

EDMAN, K. A. P. The binding of zinc to glycerol-extracted muscle, and its relaxing effect. *Acta physiol. scand.* 1959. *46*. 209—227. — The binding of zinc to glycerol-extracted rabbit psoas muscle has been correlated with the zinc + ATP-induced relaxation. The minimum amount of zinc that must be absorbed by the glycerol-extracted fibres for complete isometric relaxation is about 3—4 mmoles per kg dry weight, which is close to the amount lost during glycerol-extraction. The extracted fibres are able to absorb zinc in great excess of the concentration necessary for complete relaxation. The pH-dependence of the contraction-inhibiting effect of zinc and of the binding of zinc in high concentrations to the muscle shows that groups with a pK-value of about 7, probably imidazole groups, are involved in both cases. The finding of an additive synergism between the effects of zinc and mersalyl suggests that also SH-groups may be involved in the functionally relevant coupling of zinc to the contractile element.

Earlier publications (EDMAN 1958, 1959 a, 1959 b) have shown that zinc in the presence of ATP[1] (or UTP or ITP) induces relaxation of glycerol-extracted muscle fibre bundles. The isometric relaxation is completely reversible and is obtained even with low bath concentrations of zinc (0.0125 mM) and ATP (0.08 mM).

Several earlier observations (EDMAN 1958, 1959 b) have demonstrated that the relaxing effect is probably dependent upon an accumulation of zinc in the fibre proteins. It has therefore been the purpose of the present investigation to determine the concentration of zinc within the extracted fibres at different concentrations of zinc in the medium. For comparison, the investigation also includes determinations of zinc in fresh muscle. In order to elucidate the point of attack of zinc in the contractile system the binding of zinc to the fibres has been studied at varied pH and after blocking of SH-groups. Furthermore, the interaction of zinc and mercury on the contractility of the fibre bundles has been determined in order to demonstrate whether or not the effects of zinc and mercury are additive.

Methods.

Rabbit psoas muscle was used. The glycerol-extraction was done according to an earlier description (EDMAN 1957).

The qualities of the chemicals used were as follows: Chloral hydrate, mersalyl and Veronal (diethylbarbituric acid) were according to Ph. S. XI. For analysis of the diethylbarbituric acid, see EDMAN (1958). Tris(hydroxymethyl)aminomethane (disodium salt, Sigma) was purified before use by triple recrystallization in borosilicate glass. ATP, dibarium salt, Pabst lot. no. 129 (for analysis, see EDMAN 1958) was used. The rest of the chemicals used were of pro analysi quality.

The pH-determinations were performed with a glass electrode. The experiments were carried out at room temperature (20—22° C). If not otherwise stated, the concentrations given correspond to the total concentration in the bath. In calculation of the concentrations of free magnesium, zinc and ATP, the same stability constants (log K) were used as earlier (EDMAN 1959 a), viz. 4.00 for the MgATP complex and 4.76 for the ZnATP complex.

A. *Contraction experiments.*

The technique for isometric recording with glycerol-extracted fibre bundles has been described (EDMAN 1957). The length of the fibre

[1] The following abbreviations will be used: ADP, adenosinediphosphate; ATP, adenosinetriphosphate; EDTA, ethylenediamine tetraacetate; PCMB, p-chloromercuribenzoate; Tris, tris(hydroxymethyl)aminomethane.

bundles was 10 mm and the cross section 70—122 × 136—220 μ. The initial tension applied to the fibre bundles before contraction was 22.2 mg. The deviation from strict isometry was < 0.2 % of the length of the fibre bundles.

The medium used was a potassium chloride solution with pH 7.30 containing 10 mM diethylbarbituric acid, 100 mM potassium and 1 mM magnesium. Hereafter this solution will be called "veronal buffer". In some experiments, where the pH was varied, a Tris-acetate buffer solution (FRIEDEN and ALBERTY 1955) with the following composition was used: potassium chloride 100 mM, magnesium chloride 1 mM, acetic acid 10 mM; Tris(hydroxymethyl)aminomethane was added to the pH wanted.

The water for washing and for preparation of the solutions was double distilled in borosilicate glass.

B. *Analysis of zinc, calcium and magnesium in muscle tissue.*

The determination of zinc, calcium and magnesium in fresh and in glycerol-extracted muscle tissue was carried out largely according to the method described by HAMM (1955).

Solvents and reagents:

Veronal and Tris-acetate buffers: the same as those described above but ***without magnesium.*** The buffer solutions were analyzed quantitatively for zinc by means of microanalytical technique (page 213).

Zinc chloride solution (used for incubation of muscle tissue in the zinc uptake studies): 1 mM stock solution was prepared by dissolving $ZnCl_2$ in buffer solution (veronal or Tris-acetate). The zinc concentration in the stock solution was checked by EDTA titration.

Solutions of EDTA, chloral hydrate, potassium cyanide and ammonia-ammonium chloride were prepared according to HAMM (1955).

Eriochrome black indicator: 1 percent solution of Eriochrome black (Tamm, Sweden) in diethanolamine (Hopkin & Williams, Ltd.).

Cation exchanger: Dowex 50, × 8, 50—100 mesh.

Hydrochloric acid: redistilled in borosilicate glass.

Water: The water for washing the vessels and for preparation of reagents and standard solutions for analysis of metals in muscle tissue was distilled once and had a specific conductance of approximately 1×10^{-6} ohm^{-1} cm^{-1}. The water used in preparation of the veronal buffer and the Tris-acetate buffer was double distilled in borosilicate glass.

Ion-exchanger column: A glass tube 120 mm in length and with an internal diameter of 10 mm was sealed at one end to a capillary tube provided with a ground stopcock and at the other (upper) end to a tubing 70 mm in length and with an internal diameter of 25 mm. A plug of firmly compressed borosilicate glass wool in the lower end of the

tube served as support for the column. The resin was packed to a height of about 40 mm and protected by another plug of glass wool applied to the upper end of the column. Before being used the first time the column was washed with 6 M hydrochloric acid until no iron could be demonstrated in the eluate by extraction with thiocyanate-amylalcohol, *i. e.* < 0.1 μg Fe^{+++}/ml. Finally, it was washed with distilled water.

All vessels were rinsed with 6 M hydrochloride acid and then with distilled water before use.

Titration: A 500 μl Agla syringe was used. The titration was done in an 8 cm high borosilicate glass vessel with a volume of 7 ml. The Agla syringe was fixed horizontally, and an angled steel cannula was mounted on the syringe with its tip below the fluid level in the titration vessel. The solution was stirred by means of a glass rod with a flattened and angled lower tip.

The dry weight of the muscle material was determined by desiccation at + 105° C for 24 hours, and the nitrogen content by means of micro-Kjeldahl technique.

Combustion of the muscle tissue was carried out in a muffle furnace at + 550° C for 4—5 hours. Open platinum dishes were used in this procedure; these were placed in a covered quartz box in the furnace during the combustion.

The treatment of the ash, the separation on the cationic resin and the complexometric titration of zinc and magnesium + calcium with EDTA were done according to the technique described by HAMM (1955). In the titration procedure the zinc was first masked with cyanide, whereafter calcium + magnesium was determined by EDTA titration at pH 10. After demasking of the zinc with chloral hydrate the titration was continued for determination of zinc. For a complete demasking of zinc it proved necessary to add four times the amount of chloral hydrate used by HAMM. Calcium was determined by means of a Beckman DU flame photometer[1]. The magnesium concentration was computed by subtracting the concentration of calcium from the total concentration of calcium + magnesium.

The analytical procedure was checked by applying definite quantities of zinc (6.18 mg and 0.168 mg) and calcium (2.00 mg and 0.24 mg) to the ion-exchanger column and analyzing the eluates as just described. The recovery was 100 ± 1 per cent in both cases.

1. *Analysis of fresh muscle.*

Psoas muscle from rabbit was used. Immediately after exsanguination of the animal the psoas muscle, deprived of its fascia, was removed from the animal and was crushed to a pulp in an agate mortar. This was followed by determination of dry weight and nitrogen as well as zinc, calcium and magnesium. In each test 2.4—2.6 g fresh muscle pulp was used.

[1] Carried out by the Analytical Department of the University of Uppsala.

2. *Analysis of glycerol-extracted muscle.*

Glycerol-extracted fibre bundles from rabbit psoas were thoroughly ground to a pulp in buffer solution (veronal or Tris-acetate) in an agate mortar. After this treatment the muscle mass consisted of undifferentiated brei, single fibres and fragments of fibre bundles. The muscle tissue was then washed with buffer solution (approximately 25 g wet muscle pulp in 550 ml buffer solution) by gentle stirring for 15 min. Two such washings were carried out, followed by 30 minutes' centrifugation (3,500 r. p. m.). The pulpified and washed muscle tissue was divided into approximately equal portions, which corresponded to 0.17—1.07 g dry weight in the different experiments. One portion was used immediately for determination of zinc, calcium and magnesium; the rest was used for determination of the uptake of zinc by the muscle tissue as described under Results I 2, 3 and 4.

Because of the great accumulation of zinc in the muscle tissue it was necessary to use large volumes of bath solution in the experiments where the absorption of zinc to the muscle tissue was studied in order to avoid a marked decrease in the zinc concentration in the bath during the incubation. With concentrations of zinc in the bath of $\leq$ 0.1 mM the volume was 4000 ml, with 0.2 mM zinc the volume was 2000 ml and with higher zinc concentrations 1000 ml. The bath solution was stirred gently with a glass rod. After incubation was completed (for the incubation times, see Results), the bath solution was filtered off and the muscle pulp was kept for analysis. At the lowest zinc concentrations (0.001 and 0.01 mM) zinc analysis was carried out on the bath solution after finished incubation. In other cases the final zinc concentration in the bath solution was computed by subtracting the quantity of zinc taken up by the muscle tissue from the quantity in the bath before the incubation.

C. *Microanalysis of zinc.*

The determinations of zinc in the buffer solutions and in the bath solutions used in certain of the zinc uptake experiments were performed spectrophotometrically after dithizone-carbontetrachloride extraction of zinc according to SANDELL (1950)[1]. To prevent foaming during the extraction procedure, it was necessary to destroy the surface-active substances in the solution. Therefore, 10—30 ml of the solution was evaporated almost to dryness in a flask of quartz glass and then boiled for 2—3 min with 0.5 ml aqua regia. After addition of 1 ml hydroxylamine hydrochloride, the sample was diluted again to the desired volume. Before the extraction with dithizone, the pH was adjusted to 4.75 with an acetate buffer solution; the addition of sodium thiosulphate was omitted.

[1] These analyses were carried out by the Analytical Department of the University of Uppsala.

Table I.

Concentrations of zinc, calcium and magnesium in fresh and glycerol-extracted muscle tissue from rabbit psoas.

		Fresh rabbit psoas muscle. Two samples from each of three animals						Glycerol-extracted rabbit psoas muscle. Ten preparations
		I		II		III	Mean between the 3 animals	
Zinc	mmoles per kg dry tissue	3.4 2.9	*3.2*	3.0 3.5	*3.3*	3.3 2.7	*3.0*	3.2 ±0.1	2.4±0.4
Calcium		3.1 3.4	*3.3*	3.3 3.4	*3.4*	3.8 4.2	*4.0*	3.6 ±0.2	6.4±0.5
Magnesium		45.1 43.9	*44.5*	42.5 49.8	*46.2*	49.2 48.2	*48.7*	46.5 ±1.2	11.9±0.9
Water	Percent of dry tissue	75.93 75.88	*75.91*	76.39 76.40	*76.40*	75.78 75.92	*75.85*	76.05±0.17	—
Nitrogen		13.26 13.75	*13.51*	13.10 13.06	*13.23*	14.90 14.84	*14.87*	13.87±0.51	13.6±0.2

The values to the left in the columns I—III represent single analyses, the italicized values to the right are means.

Results.

I. *Analysis of zinc, calcium and magnesium in muscle tissue.*

The purpose of these investigations was to establish the normal concentration of zinc in fresh and in glycerol-extracted muscle and further to determine the ability of the extracted muscle tissue to bind zinc at varied zinc ion concentration in the medium, at varied pH and in the presence of blocked SH-groups. The studies also included analysis for calcium and magnesium.

1. *Analysis of fresh and glycerol-extracted muscle.*

Table I summarizes the results of analyses for zinc, calcium and magnesium in fresh muscle and glycerol-extracted, washed muscle tissue from rabbit psoas.

As may be seen, in fresh muscle the calcium concentration is approximately equal to the zinc concentration, while the magnesium concentration is 15 times as high. According to HAMM (1955) fresh beef muscle contains 2.3 mmoles zinc, 4.9 mmoles calcium and 40.6 mmoles magnesium per kg dry weight. KOGA (1934) reported 1.8 mmoles zinc per kg dry dog muscle, and KOCH, SMITH, SHIMP and CONNOR (1956) 1.1 mmoles zinc per kg wet human muscle, *i. e.* about 4.4 mmoles per kg dry material. According to VALLEE and ALTSCHULE (1949) voluntary muscle of man and other vertebrates contains 50 mg zinc per kg wet tissue, which corresponds to about 3 mmoles per kg dry weight.

After the glycerol-treatment and washing of the muscle tissue with veronal buffer solution as described (Methods B, 2) the concentration of zinc is only slightly lower than in the fresh muscle. The magnesium concentration is also lower in the extracted and washed muscle, while the calcium concentration is higher than in the fresh muscle.

In a comparison between the metal concentrations in fresh and glycerol-extracted muscle it must be kept in mind that the muscle tissue decreases in weight during the extraction. For instance, according to VARGA (1950), WEBER and PORTZEHL (1954) and HASSELBACH (1957), 20—30 percent of the protein content is lost. In the present investigation two experiments demonstrated a weight loss of 24 and 30 percent respectively. A 30 percent weight loss would imply that 53 percent of the zinc and 18 percent of the magnesium are retained in the fibres after the extraction, and that the calcium is increased by 24 percent. The increase in calcium is surprising, since the calcium concentration in the extraction and washing solutions was only 0.0045 mM, and it is improbable that any calcium contamination occurred during the experiments. HASSELBACH (1957) found that 85 percent of the calcium and 10 percent of the magnesium were retained in the insoluble structures when fresh homogenized muscle was exhaustively treated with 100 mM potassium chloride solution.

If a 30 percent weight loss is assumed, it follows from Table I that about 2 mmoles zinc must have been lost per kg dry glycerol-extracted muscle tissue during extraction.

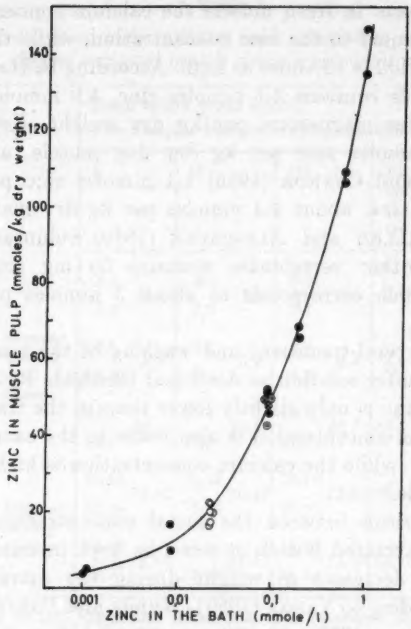


Fig. 1. Binding of zinc to glycerol-extracted muscle tissue at pH 7.3.

Medium: veronal buffer solution without magnesium; in certain experiments (○) Tris-acetate buffer solution was used instead.

Incubation time: 60 min, excepting ⊙ (30 min) and ⊗ (120 min).

Nitrogen concentration: 13.24 ± 0.30 percent of dry muscle weight.

Each symbol represents one experiment. Curve fitted by sight. Note that the abscissa has a logarithmic scale.

2. *Accumulation of zinc in the glycerol-extracted muscle with variation of the zinc concentration in the medium.*

As demonstrated earlier (EDMAN 1958, 1959 b) the contraction-inhibiting and relaxing effect of zinc is probably due to an accumulation of zinc in the glycerol-extracted muscle fibres. It is therefore of interest ot determine the capacity of the glycerol-extracted muscle tissue to bind zinc when the zinc ion concentra-

tion in the medium is varied. Fig. 1 illustrates the results of such an investigation. The medium consisted of veronal buffer solution without magnesium, and the zinc concentration was varied over a wide range (0.001—1 mM).

The incubation time for the muscle tissue in the zinc solution was varied between 30, 60 and 120 min in the experiments with 0.1 mM zinc in the bath. Thirty minutes' incubation proved sufficient to permit maximum uptake of zinc into the muscle tissue. An incubation time of 60 min was used with the rest of the zinc concentrations in order to ensure that equilibrium was reached between the homogenized muscle tissue and the bath with respect to zinc. Some experiments (0.025 mM zinc) were performed with Tris-acetate buffer solution (without magnesium) as medium. The results of these experiments did not deviate notably from the results obtained when the medium was buffered with veronal. According to NEURATH (1955) both veronal and Tris have only a slight capacity for zinc-complexing and are therefore both suitable for use as buffers in experiments of the type described here.

It is clearly evident from Fig. 1 that zinc has a great affinity for the fibre proteins. With 0.001 mM zinc in the bath the concentration in the muscle tissue is about 5 mmoles per kg dry weight as opposed to 2.4 mmoles before the incubation (Table I). With as high as 1 mM zinc concentration in the medium the muscle tissue is still unsaturated with zinc. Higher concentrations could not be studied without exceeding the solubility product for $Zn(OH)_2$.

As shown in an earlier publication (EDMAN 1959 b), 0.0025 mM free zinc ion concentration in the bath is necessary for complete isometric relaxation of glycerol-extracted fibres in the presence of ATP. According to Fig. 1, this free zinc ion concentration corresponds to about 6 mmoles zinc per kg dry fibre weight on condition that the uptake of zinc by the fibres is not changed by the presence of ATP. Evidently, the muscle fibres are able to accumulate zinc in concentrations far exceeding the threshold concentration needed for total relaxing effect.

3. *Binding of zinc to the muscle tissue after blocking of the sulfhydryl groups.*

The medium consisted of Tris-acetate buffer solution with a pH of 7.3. p-chloromercuribenzoate (PCMB) in two different con-

Table II.

Effect of SH-blocking with p-chloromercuribenzoate (PCMB) on the binding of zinc to glycerol-extracted muscle tissue.

Series of experiments	Concentration of zinc in glycerol-extracted rabbit psoas muscle (mmoles Zn/kg dry weight) after equilibration with 0.025 mM zinc in the bath			Nitrogen concentration (percent of dry muscle weight)
	Controls without PCMB	Pretreatment with 1 mM PCMB	Pretreatment with 3 mM PCMB	
I	16.7 *16.7* 16.6	15.5 *16.7* 17.8	—	12.76
II	19.6 *19.7* 19.8	—	18.9 *18.1* 17.2	
III	22.4 *21.8* 21.1	20.2 *20.1* 19.9	—	13.12

The values to the left in the columns correspond to single experiments, the italicized values to the right are means.

Series I and II: PCMB omitted in bath after pretreatment.
Series III: PCMB (1 mM) in bath also during incubation with zinc.

centrations, 1 mM and 3 mM, was used as SH-blocking agent. The muscle tissue (0.2000—0.3052 g dry weight) was first incubated in PCMB solution (1000 ml) for 60 min and thereafter in a bath consisting of 0.025 mM zinc (4000 ml) for 60 min (Table II, series I and II). In two experiments (Table II, series III) the zinc solution also contained PCMB in the same concentration that had been used in the pretreatment with PCMB. Control experiments were carried out in parallel, with the same preparation of the muscle tissue but in the absence of PCMB. The molar amounts of PCMB used were in great excess of the accessible sulfhydryl groups, amounting to 0.07 mmoles per g dry muscle weight (BENNETT and WATTS 1958). Since the affinity of mercury for the SH-groups is considerably greater than that of zinc (GURD and WILCOX 1956) it is probable that even with the low PCMB concentration there was a great excess of PCMB beyond that necessary to prevent zinc, in the concentration investigated, from combining with SH-groups.

As shown in Table II, the treatment with PCMB does not

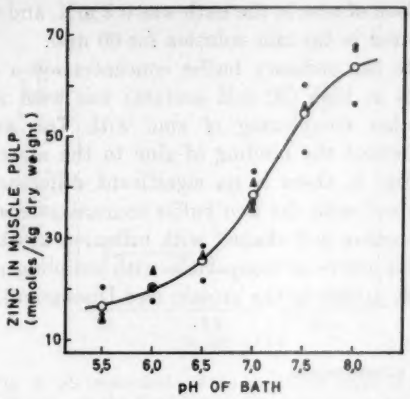


Fig. 2. pH-dependence of binding of 0.2 mM zinc to glycerol-extracted muscle tissue.

Medium: Tris-acetate buffer solution without magnesium;

●: 10 mM acetate concentration.
▲: 30 mM acetate concentration.

Mean values: ○.
Incubation time: 60 min.
Nitrogen concentration: 13.71 ± 0.14 percent of dry muscle weight.

cause any distinct change in the uptake of zinc by glycerol-extracted fibres. This means that the main part of the zinc present can combine with other groups in the protein than the SH-groups. It should be noted that the concentrations of zinc in the fibre tissue in these experiments were only 3 times higher than the threshold concentration for complete isometric relaxation (see page 217).

4. *pH-dependence of the binding of zinc to muscle tissue.*

Fig. 2 shows the binding of zinc to glycerol-extracted muscle tissue with variation of the pH in the bath from 5.5 to 8.0. The Tris-acetate buffer solution without magnesium was used as medium. The pH was determined by glass electrode in the beginning and at the end of the incubation of the muscle tissue. The

total concentration of zinc in the bath was 0.2 mM, and the muscle pulp was incubated in the zinc solution for 60 min.

In addition to the ordinary buffer concentration a concentration three times as high (30 mM acetate) was used in order to determine whether complexing of zinc with Tris and acetate noticeably influenced the binding of zinc to the muscle protein. As shown in Fig. 2, there is no significant difference between the values obtained with the two buffer concentrations used. The pH-dependence curve is S-shaped with inflexion at about pH 7. The shape of the curve is compatible with coupling of the zinc ions to imidazole groups in the protein (see Discussion).

II. *Contraction experiments.*

1. *pH-dependence of the contraction-inhibiting effect of zinc.*

It is of interest to know if the contraction-inhibiting effect of zinc is dependent on pH as is the binding of zinc to the glycerol-extracted fibres just demonstrated. The experiments were performed in two different ways: 1. determination of the contraction-inhibiting effect of a definite zinc concentration at varied pH in the medium; 2. determination of the zinc concentration necessary for a definite inhibition of the contraction at varied pH. The Tris-acetate buffer solution, containing 1 mM magnesium, was used as medium. The fibre bundles were pretreated with zinc for one hour before the start of the contraction; the zinc concentration during the pretreatment was the same as the total concentration of zinc present in the bath during the contraction. The contraction was induced by 0.4 mM ATP. The tension 5 min after the start was used as final tension, since a practically constant level had then been attained.

Fig. 3 shows the contraction-inhibiting effect of 0.0125 mM zinc on the isometric contraction of glycerol-extracted fibre bundles at varied pH in the bath. With the zinc concentration used the final isometric tension was inhibited by about 60 per cent at pH 8.0. As is seen, the inhibiting effect decreases with a decrease in pH; no inhibition of the contraction could be demonstrated when the pH was decreased to 7.5.

In the following experiments the concentration of zinc in the bath was selected so that a definite and almost complete inhibi-

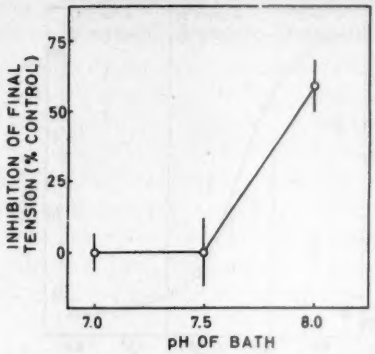


Fig. 3. pH-dependence of the inhibiting effect of 0.0125 mM zinc on the final tension in isometric contraction.

Medium: Tris-acetate buffer solution containing 1 mM magnesium. Contraction induced by 0.4 mM ATP. Each symbol represents the mean effect obtained with 4 tests and 4 controls. The standard error of the means is indicated with a bar.

tion of the final tension was attained at varied pH within the pH-range 6.0—8.0. The total concentrations of ATP (0.4 mM) and magnesium (1 mM) were kept constant. The concentration of free magnesium[1] varied between 0.68 and 0.88 mM in the different experiments, the concentration of free ATP between 0.014 and 0.048 mM. The suitable zinc concentration at the respective pH was first tested out preliminarily and then verified by 4 pairs of experiments; each pair consisted of one test (with zinc) and one control (without zinc). The inhibiting effect of zinc at the different pH-levels studied was 97 $\pm$ 0.3 percent of the final tension. The results are summarized in Fig. 4. As is seen, the zinc needed in the bath for reaching a definite effect, and consequently a definite concentration of zinc bound to functionally important sites, decreases with increasing pH; the pH-dependence curve has the form of a reversed S with inflexion at about pH 6.7. Within the concentration range of free zinc existing in the different experiments the uptake of zinc by the fibres is roughly linear with the

[1] For the stability constants of the zinc and magnesium complexes of ATP, see Methods. As is evident from the titration measurements of MARTELL and SCHWARZENBACH (1956), and WEITZEL and SPEHR (1958), the stability constants may be used unchanged over the pH-interval 6—8.

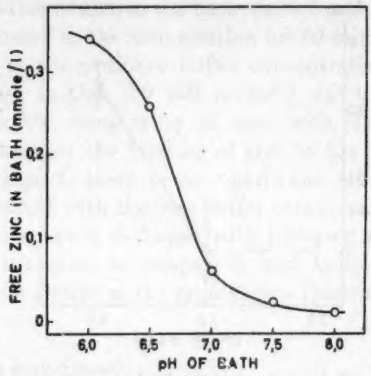


Fig. 4. Relation between pH of the bath and the concentration of free zinc necessary for 97 ± 0.3 percent inhibition of the final isometric tension.

Medium: Tris-acetate buffer solution containing 1 mM magnesium. Contraction induced by 0.4 mM ATP. Each symbol represents 4 tests and 4 controls.

concentration of zinc ions in the bath, as may be deduced from the values in Fig. 1. Therefore, the pH-dependence curve in Fig. 4 approximately illustrates the inverse pH-dependence of the coupling of zinc to *functional* sites in the fibre protein. Thus, it is evident that the same kind of pH-dependence exists for the functionally relevant binding of zinc as was demonstrated in the zinc uptake studies where zinc was bound to the fibres in great excess. According to Fig. 4, the pK-value of the functionally important groups involved in the coupling of zinc to the fibre protein is approximately 6.7. The results will be further discussed later.

2. *Additive effects of zinc and mercury.*

As demonstrated earlier (PORTZEHL 1952: mersalyl; EDMAN 1958: $HgCl_2$), mercury causes relaxation of glycerol-extracted muscle fibres in the presence of ATP. The following experiments were performed for the purpose of investigating whether the contraction-inhibiting effects of mercury and zinc are additive.

The veronal buffer solution (pH 7.3), containing 1 mM magnesium, was used as medium. The fibre bundles were pretreated

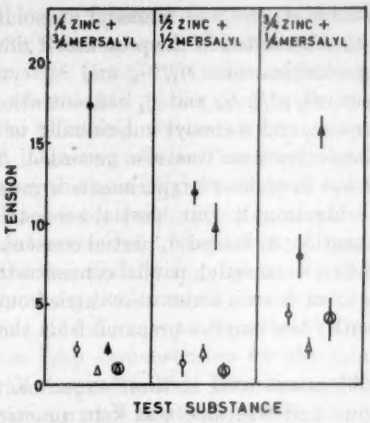


Fig. 5. Additive effects of zinc and mersalyl on the contractility of glycerol-extracted fibre bundles.

Ordinate: tension in mg per 100 μ fibre bundle circumference.
Medium: veronal buffer solution containing 1 mM magnesium.

Contraction induced by 0.4 mM ATP in the presence of:

○: 0.025 mM (= full conc.) zinc,
△: 0.022 mM (= full conc.) mersalyl,
Ⓐ: partial concentration ($^1/_4$, $^1/_2$ or $^3/_4$) of zinc + partial concentration ($^3/_4$, $^1/_2$ or $^1/_4$) of mersalyl,
●: partial concentration of zinc alone,
▲: partial concentration of mersalyl alone.

Each symbol represents the mean of 3 experiments. The standard error of the means is indicated with a bar if exceeding the size of the symbols.

with the metal (in the same concentration as used during the contraction) for one hour before the start of the experiment. Contraction was induced by changing to a solution of the metal + 0.4 mM ATP and was recorded for 5 min, after which constant tension had practically been reached. The concentrations of zinc alone (0.025 mM) and mersalyl alone (0.022 mM) were selected so that about the same, low final tension was obtained, corresponding to an almost complete inhibition of the contraction.[1] With these

[1] With 0.025 mM zinc some of the inititial tension of the fibre bundle disappeared immediately after the addition of ATP. Thereafter the bundle contracted and finally reached a level that exceeded the initial tension. This difference in effect at the beginning and at the end of contraction was probably due to the fact that the zinc concentration in the fibres decreased during the contraction course because of complex formation between zinc and ATP in the medium (EDMAN 1958).

"full" concentrations of zinc and mersalyl as point of departure the tension was then recorded in the presence of zinc and mersalyl in the following combinations: 1/4, 1/2 and 3/4 concentration of zinc with, respectively, 3/4, 1/2 and 1/4 concentration of mersalyl. The tension with zinc and mersalyl individually in the aforementioned partial concentrations was also recorded. The investigation was carried out in series of experiments arranged as follows: 1. zinc, full concentration; 2. zinc, partial concentration; 3. mersalyl, full concentration; 4. mersalyl, partial concentration; 5. zinc, partial concentration + mersalyl, partial concentration. The different experiments in each such series were carried out in immediate succession and with fibre bundles prepared from the same original bundle.

With the combinations used in these experiments the relation between total zinc and free zinc was kept practically constant, deviating only between 4.01 and 3.86. If mersalyl is bound to ATP, the percentage of free mersalyl would similarly be constant at the different total concentrations of mersalyl that were used due to the relatively high ATP concentration.

The results are summarized in Fig. 5. With all three combinations of mersalyl and zinc, as may be seen, the final tension was of the same magnitude as when only zinc or only mersalyl were used in full concentrations. It is therefore probable that the contraction-inhibiting effects of zinc and mersalyl are additive. This implies that zinc and mersalyl probably act on the same sites in the contractile system.

Discussion.

The binding of metal ions to proteins has been comprehensively treated in recent reviews by KLOTZ (1953) and GURD and WILCOX (1956), and, concerning the binding of zinc in particular, in a review by WEITZEL (1956). The problem is complicated. However, many observations have shown that imidazole groups and probably also SH-groups are important as ligands for the zinc ion.

The pH-dependence demonstrated for the binding of zinc to the glycerol-extracted fibres shows that groups in the fibre proteins with a pK-value of about 7 are involved. This is consistent with coupling of zinc to imidazole groups, the pK-value of which

is about 7 at the experimental conditions used here (KIRBY and NEUBERGER 1938; TANFORD and WAGNER 1953; EDSALL, FELSENFELD, GOODMAN and GURD 1954). For technical reasons higher concentrations of zinc had to be used in these pH-dependence studies than the threshold concentration of zinc necessary for full contraction-inhibiting effect. Therefore, the functionally relevant fraction of the zinc might have been swamped. However, the same pH-dependence (with inflexion at pH 7) was obtained even for the contraction-inhibiting effect of zinc. Hence, if one assumes that the functionally important site of binding of zinc is built up of nothing but common amino acids, it would seem that an imidazole side chain is an important part of it. A similar effect of pH has been demonstrated for the interaction of zinc with enolase (MALMSTRÖM and WESTLUND 1956) and yeast-invertase (MYRBÄCK and WILLSTAEDT 1958).

There is no definite evidence for involvement of SH-groups in the contraction-inhibiting effect of zinc. However, the fact that additive synergism exists between zinc and mersalyl makes it conceivable that SH-groups also partake in the functionally important binding of zinc. As pointed out earlier, the additive synergism implies that zinc and mersalyl probably act on the same sites in the protein. In view of the high affinity of mercury for the SH-groups as demonstrated with small molecules (STRICKS, KOLTHOFF and HEYNDRICKX 1954), it therefore seems reasonable that SH-groups are involved in the functionally relevant coupling of mersalyl and, consequently, also in the functionally relevant coupling of zinc to the contractile protein. However, mercury must not necessarily be assumed to react with sulfhydryl groups, since a specific binding of mercury to protein molecules may occur without presence of any cystein, as demonstrated for enolase (WARBURG and CHRISTIAN 1942, MALMSTRÖM, KIMMEL and SMITH 1959) and myoglobin (KENDREW 1958; KENDREW, BODO, DINTZIS, PARRISH, WYCKOFF and PHILLIPS 1958). In any case, the pH-dependence demonstrated for the contraction-inhibiting effect of zinc is not compatible with a simple SH-blockade. Thus, if SH-groups at all partake in the functionally important binding of zinc to the contractile protein, other groups, probably imidazoles, must also be involved.

In glycerol-extracted fibres the minimum concentration of zinc necessary for complete relaxation is about 6 mmoles per kg dry weight (at 0.0025 mM free zinc ion concentration in the medium),

i. e. about 3—4 mmoles per kg must be absorbed in addition to the zinc concentration originally present in the glycerol-extracted fibres. It is probable that only a fraction of this zinc is effective, since, among other things, some zinc is probably bound to non-contractile structures constituting about $^1/_3$ of the fibre weight (HASSELBACH 1957, WEBER and PORTZEHL 1954). The physiological concentration of zinc in skeletal muscle is relatively high, about 3 mmoles per kg dry weight. It is similarly probable that only a fraction of this zinc plays a part in the contractile process. However, it is interesting to note that the amount of zinc that must be taken up by the glycerol-extracted fibres for complete relaxing effect is close to the amount (about 2 mmoles per kg dry weight) lost during the extraction.

This investigation was supported by grants from the Medical Faculty of the University of Uppsala and the Magnus Bergvall Foundation.

Part of the investigation was performed at the Analytical Department of the University of Uppsala. I wish to thank Professor FOLKE NYDAHL of the Analytical Department and Docent BO MALMSTRÖM of the Biochemical Department for their advice and criticism.

My thanks are also due Mrs. BIRGIT ANDERSSON for valuable technical assistance.

References.

BENNETT, H. S. and R. M. WATTS, The cytochemical demonstration and measurement of sulfhydryl groups by azo-aryl mercaptide coupling, with special reference to mercury orange. General Cytochemical Methods (Danielli, J. F., ed.) *Academic Press, New York.* 1958. *1.* 317—374.

EDMAN, K. A. P., On the contraction of glycerol-extracted muscle fibre bundles under highly isometric conditions. *Acta physiol. scand.* 1957. *41.* 229—254.

EDMAN, K. A. P., The effect of zinc and certain other bivalent metal ions on the isometric tension development of glycerol-extracted muscle fibre bundles. *Acta physiol. scand.* 1958. *43.* 275—291.

EDMAN, K. A. P., Relaxation and visco-elastic behaviour of glycerol-extracted muscle fibre bundles in the presence of zinc and ATP. *Acta physiol. scand.* 1959 a. *45.* 164—180.

EDMAN, K. A. P., Relaxation of glycerol-extracted muscle fibre bundles induced by zinc in the presence of ATP and other polyphosphates. *Acta physiol. scand.* 1959 b. *46.* 62—87.

EDSALL, J. T., G. FELSENFELD, D. S. GOODMAN and F. R. N. GURD, The association of imidazole with the ions of zinc and cupric copper. *J. Amer. chem. Soc.* 1954. *76.* 3054—3061.

FRIEDEN, C. and R. A. ALBERTY, The effect of pH on fumarase activity in acetate buffer. *J. biol. Chem.* 1955. *212.* 859—868.

GURD, F. R. N. and P. E. WILCOX, Complex formation between metallic cations and proteins, peptides and amino acids. *Advanc. Protein Chem.* 1956. *11.* 311—427.

HAMM, R., Über die Bestimmung von Calcium, Magnesium, Zink und Eisen in tierischem Gewebe. *Biochem. Z.* 1955. *327.* 149—162.

HASSELBACH, W., Die Bindung von Adenosindiphosphat, von anorganischem Phosphat und von Erdalkalien an die Strukturproteine des Muskels. *Biochim. biophys. Acta* 1957. *25.* 562—574.

KENDREW, J. C., Int. Congr. Biochem. 1958, Symp. No. VIII., quoted by B. G. Malmström. *Pers. comm.*

KENDREW, J. C., G. BODO, H. M. DINTZIS, R. G. PARRISH, H. WYCKOFF and D. C. PHILLIPS, A three-dimensional model of the myoglobin molecule obtained by X-ray analysis. *Nature.* 1958. *181.* 662—666.

KIRBY, A. H. M. and A. NEUBERGER, Glyoxalines: the determination of their pK values and the use of their salts as buffers. *Biochem. J.* 1938. *32.* 1146—1151.

KLOTZ, I. M., Protein interactions, The Proteins (Neurath, H. and K. Bailey, eds.) *Academic Press, New York.* 1953. *1 B.* 727—806.

KOCH, H. J., E. R. SMITH, N. F. SHIMP and J. CONNOR, Analysis of trace elements in human tissues. *Cancer* 1956. *9.* 499—511.

KOGA, A., Répartition du zinc dans les organes des animaux. *Keijo J. Med.* 1934. *5.* 97—105.

MALMSTRÖM, B. G., J. R. KIMMEL and E. L. SMITH, Quantitative amino acid composition and N-terminal sequence of yeast enolase. *J. biol. Chem.* 1959. In press.

MALMSTRÖM, B. G. and L. E. WESTLUND, The effect of pH on the interaction of enolase with activating metal ions. *Arch. Biochem. Biophys.* 1956. *61.* 186—196.

MARTELL, A. E. and G. SCHWARZENBACH, Adenosinphosphate und Triphosphat als Komplexbildner für Calcium und Magnesium. *Helv. chim. Acta* 1956. *39.* 653—661.

MYRBÄCK, K. and E. WILLSTAEDT, Studies on yeast invertase. II. Inactivation of invertase by iodine and by heavy metal ions. *Arkiv för Kemi* 1958. *12.* 203—218.

NEURATH, H., Carboxypeptidase and procarboxypeptidase. Methods in Enzymology (Colowick, S. P. and N. O. Kaplan, eds.) 1955. *2.* 77—83.

PORTZEHL, H., Der Arbeitszyklus geordneter Aktomyosinsysteme (Muskel und Muskelmodelle). *Z. Naturforschung* 1952. *7 b.* 1—10.

SANDELL, E. B., Colorimetric determination of traces of metals. *Ed. II. Interscience Publishers, New York.* 1950.

STRICKS, W., I. M. KOLTHOFF and A. HEYNDRICKX, Formation and properties of varous mercuric mercapto thioglycolates formed in reactions between mercuric mercury and thioglycolic acid. *J. Amer. chem. Soc.* 1954. *76.* 1515—1519.

TANFORD, C. and M. L. WAGNER, The consecutive constants for the association o cadmium with imidazole. *J. Amer. chem. Soc.* 1953. *75.* 434—435.

VALLEE, B. L. and M. D. ALTSCHULE, Zinc in the mammalian organism, with particular reference to carbonic anhydrase. *Physiol. Rev.* 1949. *29.* 370—388.

VARGA, L., Observations on the glycerol-extracted musculus psoas of the rabbit. *Enzymologia* 1950. *14.* 196—211.

WARBURG, O. and W. CHRISTIAN, Isolierung und Kristallisation des Gärungsferments Enolase. *Biochem. Z.* 1942. *310.* 384—421.

WEBER, H. H. and H. PORTZEHL, The transference of the muscle energy in the contraction cycle. *Progr. Biophys.* 1954. *4.* 60—111.

WEITZEL, G., Chemie und Physiologie biogener Zink-Verbindungen. *Angew. Chem.* 1956. *68.* 566—573.

WEITZEL, G. and T. SPEHR. Zink-Komplexe von Purinen. Nucleosiden und Nucleotiden. *Hoppe-Seyl. Z. physiol. Chem.* 1958. *313.* 212—226.

Acta physiol. scand. 1959. *46*. 228—233.

From the University Institute for Experimental Medical Research, Ullevaal Hospital, Oslo, Norway.

Polarographic Determination of Intravascular Oxygen Tensions in Vivo.

By

KJELL JOHANSEN and JOHN KROG.

Received 14 January 1959.

Abstract.

JOHANSEN, KJ. and J. KROG. Polarographic determination of intravascular oxygen tensions in vivo. *Acta physiol. scand.* 1959. *46*. 228—233. — The polarographic method for determination of tissue oxygen tension in vivo is discussed. Data concerning construction and characteristics of a teflon covered, catheter mounted oxygen electrode is reported.

There has lately been a good deal of interest in the use of polarographic methods for determination of oxygen tensions in various tissues.

However, most investigators have experienced difficulties in obtaining a stable enough electrode assembly allowing a continuous recording of oxygen tension in vivo.

We have, in conjunction with studies of the regional blood flow during local hypothermia, developed a teflon covered polarograph electrode for intravascular oxygen tension measurements which we have found satisfactory. Since most investigators still seem to have considerable difficulties with the practical applications of the polarographic method, we feel that it might be of some general interest to give a short report of our findings and methods.

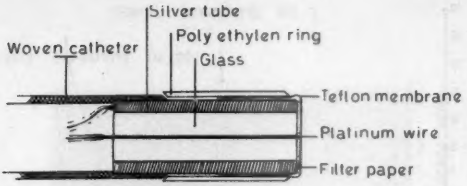


Fig. 1. Construction of catheter electrode for continuous measurement of intravascular oxygen tensions in vivo.

Construction of Electrode.

Fig. 1 shows the electrode which we have constructed and used in conjunction with intravascular oxygen tension determinations in the last years (KROG and JOHANSEN 1958). The electrode is a combined silver-silver chloride platinum electrode which is covered with a thin membrane of teflon.

Teflon is a tetrafluorethylen plastic which is very resistent to acids and can be heated above 300° C before it is destroyed. It is therefore possible to dry sterilize the material. Moreover, it is very permeable to oxygen. CLOWES (1958) registered an oxygen permeability of 16.7 ml $O_2/m^2/min$ for a membrane 0.001 inches thick as compared to 7.6 ml $O_2/m^2/min$ through a polyethylene membrane of the same thickness. The membrane is hydrophobic and therefore little subjected to adhesion of tissue proteins. The electrode shown in Fig. 1 is mounted in a heart catheter of approximately $1^1/_2$ mm in diameter. Such an assembly can easily be inserted into the larger blood vessels. The thin teflon membrane acts as a constant diffusion barrier to the oxygen in the outside media.

Fig. 2 shows a current voltage curve obtained with one of our electrodes with a plateau from 0.4 to 0.8 volt.

The described construction is advantageous in many ways. One of the most important features is that a relatively constant diffusion barrier to the oxygen in the surrounding media is facilitated. Contamination from tissue fluids is greatly diminished and a more stable electrode is obtained. Another advantage of this type of electrode is its ease of calibration which can be readily made before and after use, either in blood or saline by means of gas mixtures of known composition. (Fig. 3.)

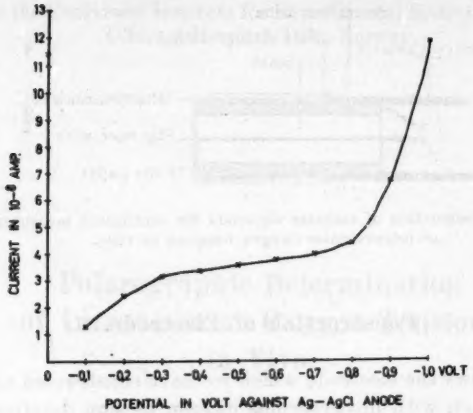


Fig. 2. Current voltage curve obtained with teflon covered combined silver platinum electrode in air.

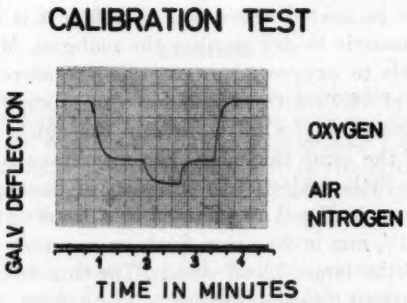


Fig. 3. Calibration curve obtained in physiological saline with teflon covered catheter electrode.

Operation Characteristics.

The catheter electrodes have been used extensively in conjunction with experiments on dogs and applied both in the aorta and in the vena cava. Oxygen determinations from the blood stream of the experimental animal have been obtained simultaneously with graded variations in the composition of the respiratory gases. (Fig. 4.)

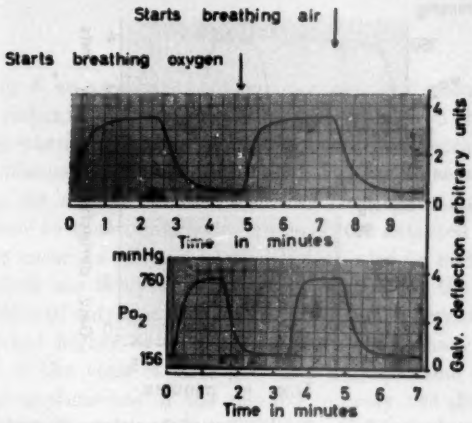


Fig. 4. Upper tracing: Polarographic oxygen recordings from aorta of a dog, breathing alternately air and oxygen.

Lower tracing: Polarographic oxygen recordings in saline, the gas tension of which has been altered between that of air and oxygen

The same electrode was used in both curves.

Fig. 4 shows recordings from a polarographic oxygen determination in the aorta of a dog when the oxygen tension in the respiratory gases was varied between 20 %—100 % oxygen. The lower tracing in the figure shows the calibration curve obtained in saline at the same temperature as that of the blood, approximately 37° C.

Fig. 5 presents the results of a stability test. This particular investigation lasted for three hours. The total drift was found to be about $\pm$ 1½ % including drift possibly due to temperature variations. The amplifications used may, without distortions to the tracings, be increased as much as 5 times above that which was utilized for the curves presented in the figures. This means that for an oxygen tension variation of 600 mm Hg one will be able to obtain a deflection on the graph of 150 mm, thus 1 mm on the scale would correspond to an oxygen tension of 4 mm Hg. Considering the drift, one should thereby be able to measure oxygen tension variations with an accuracy of about 6 mm Hg.

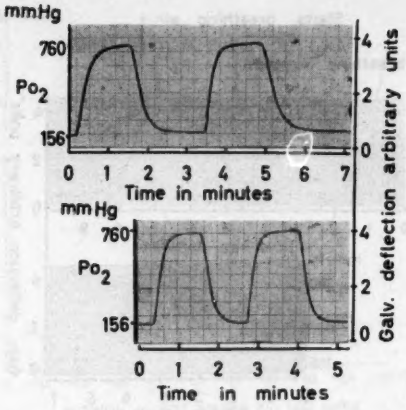


Fig. 5. Tracings obtained from a stability test of the catheter electrode described above. Upper curves obtained before and lower curves obtained after intravascular oxygen determinations lasting three hours.

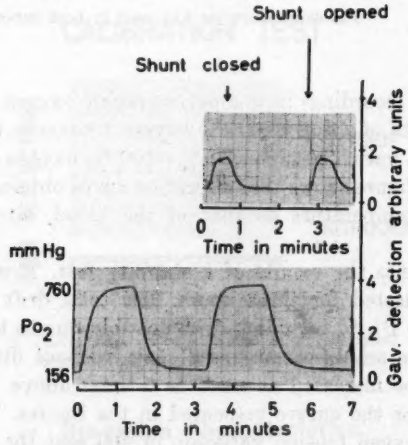


Fig. 6. Upper tracing: Polarographic determination of oxygen tension variations in vena cava with opened and closed arteriovenous shunt between the femoral artery and vein.

Lower tracing: Polarographic oxygen recordings in saline, the gas tension of which has been altered between that of air and pure oxygen.

The same electrode was used for both curves.

Application.

In Fig. 6 an application of our electrode is demonstrated. The tracing indicates the variation in oxygen tension in the vena cava of a dog when an artificial shunt between the femoral artery and vein is alternately opened and closed. It may be seen that variations in the oxygen tension resulting from shunting of arterial blood over to the venous side can easily be detected centrally in the vena cava. In this connection it may also be mentioned that the method has been found sensitive enough for the localization of the place of entrance of the renal vein, which carries blood with a somewhat higher oxygen tension than that which is found in the rest of the vena cava. Fig. 6 also indicates one of the more practical applications of the method, namely the detection and localization of arteriovenous shunts in the vascular system. As other possible uses, can be mentioned registration of oxygen consumption of different organs in situ, cardiopulmonar function studies and investigations on the effects of vasoactive drugs.

Conclusion.

The polarographic method for oxygen determination in vivo seems to open great possibilities in connection with investigations where it is of importance to follow continuously the oxygen tension variations in different organs and tissue areas in the organism. The method may thereby be a valuable aid in solving fundamental problems in physiology which hitherto have defied the attacks of the investigators, due to lack of satisfactory methods.

References.

Clowes, G. A. H., Jr. and W. E. Neville, The Membrane Oxygenator. Extracorporeal Circulation. *Charles C. Thomas Publ. Springfield.* Ill. 1958. pp. 83.

Krog, J. and K. Johansen, Construction and Characteristics of Teflon Covered Polarographic Electrode for Intravascular Oxygen Determination. Accepted for publication in *Rev. Sci. Instr.* Feb. 1959.

Acta physiol. scand. 1959. *46*. 234—239.

From the Institute of Zoophysiology, University of Oslo, Norway.

Depression of Metabolism in the Duck during Experimental Diving.

By

HARALD T. ANDERSEN.[1]

Received 18 January 1959.

Abstract.

ANDERSEN, H. T. Depression of metabolism in the duck during experimental diving. *Acta physiol. scand.* 1959. *46*. 234—239. — Diving metabolism has been studied in the domestic duck. The oxygen consumption from the lung air decreased very markedly, and the total consumption of oxygen as calculated from all stores was only some 20 % of the pre-dive level. The body temperature also dropped steadily in spite of an increased thermal insulation, due to peripheral vasoconstriction. It is concluded that the energy metabolism is greatly depressed during a quiet dive.

When mammals and birds dive they generally exhibit a series of physiological adjustments among which the following are conspicuous: The heart rate slows down in spite of muscular activity. The concentration of lactic acid in the blood increases little or not at all during diving, but rises steeply in the muscles, indicating that the muscular circulation is largely suspended when the animal is submerged. Likewise there is a marked vasoconstriction in the flippers when the seal dives.

In some diving animals, such as seals, porpoises and ducks, the oxygen stores are markedly insufficient to maintain full oxygen consumption during submergence. As these animals commonly suffer a fall in body temperature during diving and also show a

[1] Present address: Scripps Institution of Oceanography, University of California, La Jolla, California.

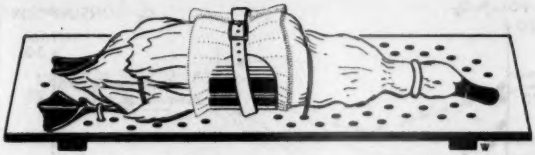


Fig. 1. Duck fastened to the board.

small excess intake of oxygen after a dive, a lowered metabolic rate during submergence is strongly indicated (SCHOLANDER 1940, IRVING, SCHOLANDER and GRINNELL 1942, SCHOLANDER, IRVING and GRINNELL 1942 a).

Most of the information available on the metabolic response to prolonged diving has been derived from experiments in which the animal was totally submerged. Because of the great cooling capacity of water, the thermal effects of the variations in tissue conductance which takes place during diving will become large. For an estimation of the heat budget, it is therefore an advantage to expose a minimum of the body surface to water. In the present investigation the duck was kept out of water, and only the head was submerged in order to induce conditions of diving. Estimates of the energy metabolism during diving have been obtained by simultaneous measurements of body temperatures and the thermal insulation of tissue.

Methods.

Diving experiments were performed on five domestic ducks weighing from 1.6 to 2.2 kg. They were fastened to a board with their necks extended (Fig. 1). In order to determine the oxygen consumption before diving, the animals were connected to a spirometer by means of a mask which fitted air-tight over the beak. The system was arranged as a closed circuit, and included air pump, flow meter and carbon dioxide absorber.

Apnoea was accomplished with a minimum of thermal disturbance by tilting the board so that only the head was submerged in water of room temperature.

To obtain samples of lung air during diving, the trachea was cannulated below the glottis, and a plastic tube, i. d. 2 mm, was inserted 10—12 cm. At suitable intervals, 10 ml of gas were withdrawn through the tube into a recipient filled with mercury and returned to the bird. After 3—4 such washings, a sample of 2 ml was taken and analyzed for oxygen and carbon dioxide in the 0.5 ml analyzer (SCHOLANDER 1947).

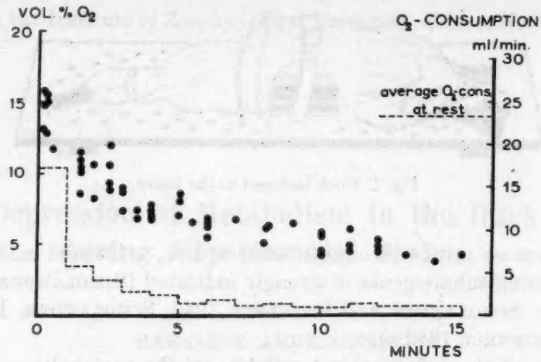


Fig. 2. Points show oxygen depletion from lung air when head of duck was submerged, data from 9 experiments.

Columns show the rate of oxygen consumption as calculated from the estimated volume of diving air, and from the average decrease in oxygen concentration per minute. The total oxygen consumption calculated from all stores would be approximately 25 % larger.

Deep body temperature was obtained by means of a thermometer inserted through the cloacum to a depth of 7 cm. Cloacal temperature was taken with a thermometer inserted 3 cm. The temperature of the thigh and the wing musculature were measured with thermocouples threaded through the tissue.

The thermal insulation of the muscles before, during and after diving was estimated using a hot wire technique: A thermocouple and an 0.5 cm long resistance wire provided with copper leads, were insulated from each other and twisted together so that the thermocouple junction could be locally heated by the resistance wire. This unit was threaded through the thigh muscles. Before the animal was submerged, the current through the hot wire was adjusted to cause a local temperature increase of 1° C when left on for 15 sec. The various heating values observed before, during and after diving relate to the circulatory heat dissipation and were compared with the heating obtained when the circulation was completely blocked by means of a tourniquet around the thigh proximal to the wires.

The heart rate was recorded by means of an electrocardiograph.

Results.

Oxygen consumption during the dive. The oxygen content of the lung air diminished at a rapid rate during the first minutes after the head was submerged, one-half of the oxygen being consumed

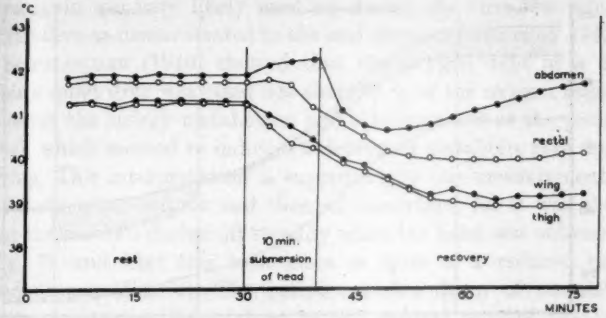


Fig. 3. Central and peripheral body temperatures recorded from a duck before, during and after submergence of head.

within 3 min (Fig. 2). As the volume of nitrogen in the lungs does not change appreciably during diving, the oxygen consumption from the lung air can be computed from the gas components and the volume of air in the lungs during the submersion. The latter may be taken as close to 300 ml or 60 ml O_2 (RICHET 1899, Vos 1934). It may be seen from Fig. 2 that the oxygen consumption from this source alone can hardly have been more than 0.6—0.4 ml per min toward the end of the dive, which is only about 2 % of the rate of the total oxygen consumption before the dive.

Body temperature. The animal commonly shivered and fluffed up the feathers during the recovery period after the head had been submerged for 10—15 min. During the dive the temperatures of the thigh and wing musculature dropped by about 1° C, and the cloacal temperature fell slightly less (Fig. 3). In contrast, there was usually a slight rise in the abdominal temperature.

In the first minutes of the recovery all temperatures fell by about 1.5° C. The deep body temperature went back to normal in about 30 min, while the rest of the body remained cool for more than two hours.

Heart rate. Before the dive the heart rate was about 110 beats per min, but when the head was submerged it slowed down gradually to about 20 beats or less after 3 min.

Thermal insulation during diving. The thermal insulation of the thigh muscles of the duck before, during and after diving may be inferred from measurements given in Fig. 4. It will be seen that the heating afforded by the hot wire during the last part

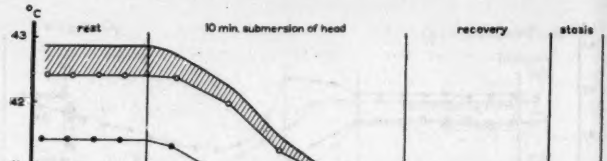


Fig. 4. Temperature and thermal insulation of a duck before, during and after submergence of head.

Lower line: Temperature of thigh musculature as indicated by unheated thermocouple.

Middle line: Temperature level obtained when the thermocouple was heated by a constant current. In the last panel marked "stasis" the circulation was blocked by a tourniquet, resulting in maximal heating.

Upper line: Maximal heating of tissue when circulation is stopped. It is here assumed that the temperature-rise produced by stasis would be the same in all phases of the experiment. The distance between the upper and middle line accordingly indicates the degree of circulation. It will be seen that toward the end of the dive, the heat dissipation from the muscles is almost as low as that of stasis.

of the dive was almost as high as that recorded when the circulation was completely blocked by means of a tourniquet ("stasis"). These results were duplicated in two repeated experiments, and they show that the thermal conductivity of this tissue decreases very markedly during diving, no doubt as a result of peripheral vasoconstriction.

Discussion.

The domestic ducks used for these experiments quietly endured an apnoea lasting for 15 min. The oxygen available in this time is mainly derived from the lung air and the blood. According to determinations by RICHET (1899) and WASTL and LEINER (1931), the oxygen content of these stores amounts to only some 80 ml in a 2 kg duck, which is less than $^1/_4$ of the oxygen required during 15 min of air-breathing. The rate of oxygen consumption from the lung air diminished greatly during diving, and parallels a similar loss of oxygen from the blood. The oxygen stores in the

myoglobin are very likely used up during the first few minutes of the dive as demonstrated in the seal (SCHOLANDER *et al.* 1942 b).

SCHOLANDER (1940) showed that the oxygen debt of a duck after a quiet dive was often less than 30 % of the oxygen required to cover the energy metabolism had this remained at the pre-dive level, which seemed to indicate a depressed metabolic rate during diving. This interpretation is supported by our measurements of the body temperatures and thermal insulation, for it was shown that the animals cooled off steadily when the head was submerged (Fig. 3), and that this took place in spite of a reduced tissue conductance (Fig. 4). The peripheral slow-down of circulation causes a conservation of heat in the central part of the body and explains the slight increase of the abdominal temperature (Fig. 3). In the recovery the increased blood flow distributes the small amount of heat produced during the dive to all parts of the body, and there is a consequent general fall in temperature in spite of improved feather insulation. Together these facts can only mean reduced heat production during submergence.

According to these findings, it is concluded that the anaerobic processes during submergence of the head did not compensate for the depressed aerobic metabolism, but left the total energy metabolism lowered.

I am greatly indebted to Professor P. F. SCHOLANDER for inspiring help during all stages of this investigation. I also wish to acknowledge the invaluable co-operation from all members of the staff of the Institute of Zoophysiology.

References.

IRVING, L., P. F. SCHOLANDER and S. W. GRINNELL, The regulation of arterial blood pressure in the seal during diving. *Amer. J. Physiol.* 1942. *135.* 557—566.

RICHET, CH., De la résistance des canards à l'asphyxie. *J. Physiol. Path. gén.* 1899. *1.* 641—650.

SCHOLANDER, P. F., Experimental investigations on the respiratory function in diving mammals and birds. Hvalraadets Skrifter, Nr. 22. 1—131. *Det Norske Videnskaps-Akademi i Oslo. Oslo,* 1940.

SCHOLANDER, P. F, Analyzer for accurate estimation of respiratory gases in one-half cubic centimeter samples. *J. biol. Chem.* 1947. *167.* 235—250.

SCHOLANDER, P. F., L. IRVING and S. W. GRINNELL, On the temperature and metabolism of the seal during diving. *J. cell comp. Physiol.* 1942 a. *19.* 67—78.

SCHOLANDER, P. F., L. IRVING and S. W. GRINNELL, Aerobic and anaerobic changes in seal muscles during diving. *J. biol. Chem.* 1942 b. *142.* 431—440.

WASTL, H. und G. LEINER, Beobachtungen über die Blutgase bei Vögeln. I. II. III. *Pflugers Arch. ges. Physiol.* 1931. *227.* 367—474.

VOS, H. J., Über den Weg der Atemluft in der Entenlunge. *Z. vergl. Physiol.* 1934. *21.* 552—578.

Acta physiol. scand. 1959. 46. 240—243.

From the Institute of Zoophysiology, University of Oslo, Norway.

A Note on the Composition of Alveolar Air in the Diving Duck.

By

HARALD T. ANDERSEN.[1]

Received 18 January 1959.

Abstract.

ANDERSEN, H. T. A note on the composition of alveolar air in the diving duck. *Acta physiol. scand.* 1959. *46.* 240—243. — The oxygen-carbon dioxide diagram obtained in two successive dives performed by domestic ducks are different in shape, the curve for the first dive shows a conspicuous S-shape, whereas the second dive performed on a high lactic acid concentration in the blood presents a straight line. This striking change may be explained as caused by a breakdown of the buffering capacity of the blood.

Many diving animals are able to endure submersion for a very prolonged period of time, and are therefore convenient subjects for the study of respiratory gas exchange during periods of arrested breathing.

The composition of alveolar air during diving, breath-holding or rebreathing may be described in a diagram where the oxygen content is plotted as abscissa and carbon dioxide as ordinate. Such diagrams have been reported for many diving animals, and the slope of the curve has been shown to be sigmoid (Fig. 1).

[1] Present address: Scripps Institution of Oceanography, University of California, La Jolla, California.

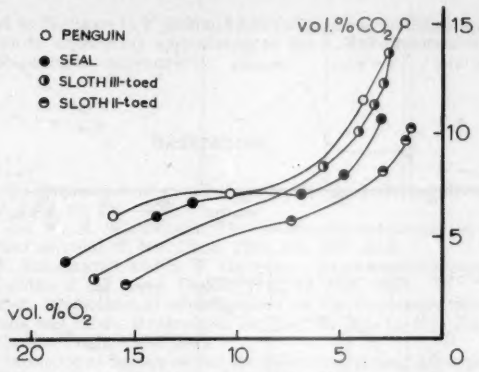


Fig. 1. Composition of alveolar air during rebreathing. Redrawn from SCHOLANDER (1940), and IRVING, SCHOLANDER and GRINNELL (1942).

SCHOLANDER (1940) pointed out that the steep rise in the sigmoid curve toward the end of a long submersion is associated with, and very likely caused by, the formation of lactic acid: When the oxygen tension in the muscles becomes zero, lactic acid is formed. Some of this acid leaks out into the circulation causing a release of carbon dioxide from the blood and tissue buffers, and very likely CO_2 also diffuses directly out from the muscles. Therefore the concentration of carbon dioxide in the lung air rises relatively steeply toward the end of the dive.

When the animals emerge, the blood is flushed with lactic acid (SCHOLANDER 1940). For several minutes a high level of lactate is present in the blood, and the buffering capacity for carbon dioxide must then be markedly diminished. In case diving was repeated during this period, one would expect the oxygen-carbon dioxide diagram to become less S-shaped.

In order to show the effect of impaired buffer capacity on the oxygen-carbon dioxide diagram, a series of experiments was carried out in which the ducks were exposed to two successive dives.

Material and Methods.

This investigation and a study of the diving metabolism in the duck were carried out at the same time. Except for the blood analyzes, the material and the methods were identical (ANDERSEN 1959).

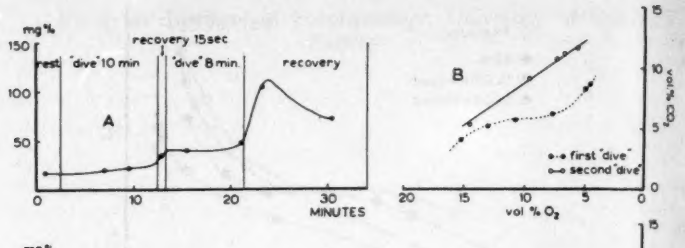


Fig. 2. A and C: Lactic acid content in the blood during two successive dives. B and D: The oxygen-carbon dioxide diagrams from the same dives.

Blood samples were taken through a small polyethylene catheter inserted approximately 5 cm into one of the veins of the wing. The blood was analyzed for lactic acid according to the colorimetric method described by BARKER and SUMMERSON (1941) and modified by STRÖM (1949).

Results and Conclusion.

Lactic acid content of the blood. In agreement with earlier findings (SCHOLANDER 1940), only a slight increase of lactic acid was observed in the blood during diving. After emerging, however, the content of lactate rose markedly.

The ducks were exposed to a second dive while the lactic acid concentration in the blood was still abnormally high (Fig. 2 A and C).

Respiratory gas exchange. The oxygen-carbon dioxide diagram of the first dive showed the well-known S-shape, the curve obtained for the second dive, however, presented an inclining, straight line (Fig. 2 B and D).

There can be little doubt that the reason for this striking alteration of the oxygen-carbon dioxide diagram is due to a decrease in the buffering capacity for carbon dioxide caused by the abnormal level of lactic acid in the blood.

I am indebted to Professor P. F. SCHOLANDER for inspiring guidance during this work. I also wish to express my appreciation to Dr. K. LANGE ANDERSEN for his ample assistance during the experiments.

References.

ANDERSEN, H. T., Depression of metabolism in the duck during diving. *Acta physiol. Scand.* 1959. *46*. 234—239.

BARKER, S. B. and W. H. SUMMERSON, The colorimetric determination of lactic acid in biological material. *J. biol. Chem.* 1941. *138*. 535—554.

IRVING, L., P. F. SCHOLANDER and S. W. GRINNELL, Experimental studies of the respiration of sloths. *J. cell. comp. Physiol.* 1942. *20*. 189—210.

SCHOLANDER, P. F., Experimental investigations on the respiratory function in diving mammals and birds. Hvalraadets Skrifter, Nr. 22. 1—131. *Det Norske Videnskaps-Akademi i Oslo.* Oslo, 1940.

STRÖM, G., The influence of anoxia on lactate utilization in man after prolonged muscular work. *Acta physiol. Scand.* 1949. *17*. 440—451.

Acta physiol. scand. 1959. *46*. 244—251.

From the Institute of Physiology, University of Helsinki, Finland.

Oxygen Consumption of Blood and Plasma and the Percentage of Reticulocytes.

By

EEVA JALAVISTO.

Received 26 January 1959.

Abstract.

JALAVISTO, E. Oxygen consumption of blood and plasma and the percentage of reticulocytes. *Acta physiol. scand.* 1959. *46*. 244—251. — Determinations of plasma and blood oxygen consumption during and after reticulocytosis due to repeated bleedings were performed on arterial blood samples taken from 9 rabbits. Plasma oxygen consumption showed no consistent relation to the degree of reticulocytosis. The oxygen consumption of whole blood followed promptly variations in the percentage of reticulocytes except for a decline in oxygen consumption often before the highest reticulocyte count was attained. This may be due to the fact that red cells, containing small remnants of the basophilic material, are counted as reticulocytes whereas the decline in oxygen consumption is probably related to the enzymatic deterioration of the citric acid cycle and the cytochrome system, which proceeds with considerable speed irrespective of the morphologic ripening of reticulocytes.

The metabolic differences between mature red cells and reticulocytes have received increasing interest in recent years. Many studies indicate an elevated concentration of various enzymes of the cells.

The oxygen consumption of red cells is mainly bound to the presence of reticulocytes (WARBURG 1909). The oxygen consumption of mature erythrocytes is almost negligible, whereas the reticulocytes have a respiratory metabolism and there exists a proportionality between percentage of reticulocytes and oxygen consumed (HARROP 1919, RAMSEY and WARREN 1933, KOMATSU 1956—57, YAMAMOTO 1935, 1936).

The rate of glycolysis is likewise found to be increased in conditions with increased percentage of reticulocytes as shown by studies of SELWYN and DACIE (1954), and HOLLINGSWORTH (1955) in haemolytic disease. The latter author found, however, that the correlation between the rate of glycolysis and percentage of reticulocytes is rather poor, most likely because the cells continue to glycolyse at a high rate after having lost their reticulum. The author, therefore, points out that the glycolytic rate may possibly give a rough index of the mean red cell age and thus indirectly of bone marrow activity. A similar relation between reticulocytes and the rate of methemoglobin reduction (MRR) in nitrite treated cells is shown by JALAVISTO (1959), JALAVISTO *et al.* (1959). The initial parallelism between the percentage of reticulocytes and methemoglobin reduction rate (MATTHIES, JUNG and ONNEN 1953) no longer holds in the posthaemorrhagic phase when the blood contains much newly formed cells but the reticulocytosis has subsided. The elevated rate of MR may under these conditions last for about 20—30 days. The plasma oxidation reduction potential which is shown to increase parallel to the development of reticulocytosis (JALAVISTO and PIHA 1952, PIHA 1956) is considered as an indication of overall metabolic activity of red blood cells. An elevated level after subsidence of reticulocytosis therefore suggests persistence of intense metabolic activity beyond the morphological ripening of the reticulocytes (PIHA 1956).

When the correlation between reticulocytes and oxygen consumption of red cells has been investigated, the declining phase of reticulocytosis has received relatively little attention. In view of the slow decrease of metabolic activity as compared with maturation of the reticulocytes, the relation between disappearance of the reticulum and oxygen consumption deserves perhaps further study.

The oxygen consumption of blood plasma is under normal conditions very small, at the limit of measurability. It probably depends on autooxidation of lipids and/or some protein com-

ponent in blood plasma (RAMSEY and WARREN 1934). Since studies with C^{14} marked acetate have shown a considerable mobility and exchange of blood lipids between cells and plasma (JAMES, LOVELOCK and WEBB 1957) the oxygen consumption of plasma in bleeding anaemia has gained added interest, particularly since it was shown by PARSONS and PARSONS (1927) that the O_2 liberated from plasma by sodium ferricyanate is increased in bleeding anaemia.

The purpose of this paper is to give data concerning the relation between oxygen consumption of blood and plasma during the development of bleeding anaemia and recovery from it.

Material and Methods.

Blood samples were withdrawn from carotid loops exteriorized prior to the experimental period in 9 rabbits. The rabbits were bled irregularly so that variations in the percentage of reticulocytes were established. The coagulation was prevented with a drop of heparin solution in the centrifuge tube used for collection of the blood sample. The haematocrit and percentage of reticulocytes were determined from the sample. After staining with brilliant cresyl blue the reticulocytes were counted according to the method of BRECHER and SCHNEIDERMAN (1950) using a Miller's ocular micrometer. Part of the blood sample was centrifuged for determination of the oxygen consumption of blood plasma. Two ml of blood or plasma were introduced into calibrated Warburg flasks of approximately 16 ml capacity and the oxygen uptake at 37° C was determined according to the standard method described by UMBREIT *et al.* (1945) with absorption of CO_2. The samples were equilibrated during 15 min and the oxygen consumption was measured after incubation for 1 and 3 hours. The oxygen consumption was expressed in μl/min/per ml packed red cells or per ml plasma, respectively. The methodical error in 32 duplicate determinations was

$$S = \sqrt{\frac{\Sigma \Delta^2}{2n}} = 0.11 \ \mu\text{l/min/ml}$$

packed red cells in the experiments on blood oxygen consumption.

Since the oxygen consumption of plasma is considered to be due to autooxidation of lipid material the rabbits were in part of the experiments injected with an antioxydant 2—3 hours prior to the withdrawal of the blood samples in order to stabilize the lipids contained in blood plasma and minimize haemolysis of cells (RÄIHÄ 1955) which would cause a burst of increased oxygen consumption of plasma (RAMSEY and WARREN 1930, 1934/35). For this purpose 25—30 mg α-tocopherol ("Ephynal", Roche) were injected intramuscularly into the rabbits. It was, however, not possible to induce any significant elevation of the level of α-tocopherol in blood and further experiments were therefore abandoned.

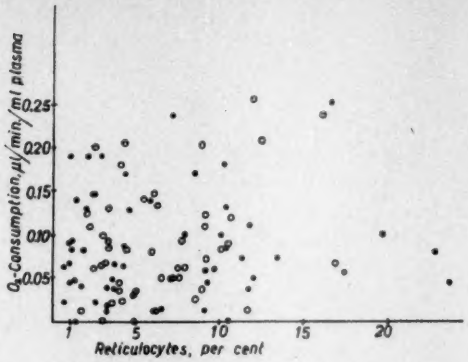


Fig. 1. Oxygen consumption of plasma and the percentage of reticulocytes.

Circles: blood from rabbits injected with α-tocopherol.
Solid circles: untreated.

Results.

Oxygen consumption of plasma. No clear relation between oxygen consumption of plasma and the percentage of reticulocytes was found. The oxygen consumption varied between 0 and 0.26 µl/min/ml plasma. Most of the values were thus within the range of methodical error. There was no significant difference between the plasma samples from untreated rabbits and from those injected with "Ephynal". The mean of the oxygen consumption in different ranges of reticulocyte percentages was the same in untreated cases, whereas it was slightly higher in plasma samples from "Ephynal"-treated rabbits when the percentage of reticulocytes exceeded 11 per cent. However, the number of determinations in this category was too small to allow any definite conclusions. The results appear in Fig. 1.

Oxygen consumption of whole blood. The oxygen consumption of blood varied between 0.56—3.65 during the 1st and 0.28—2.95 µl/min/ml packed red cells during the 3rd hour of incubation. The mean uptake increased steadily with increasing percentage of reticulocytes. No difference between "Ephynal"-injected and untreated rabbit cells is seen.

In Fig. 2 and 3 the close correlation between variations in

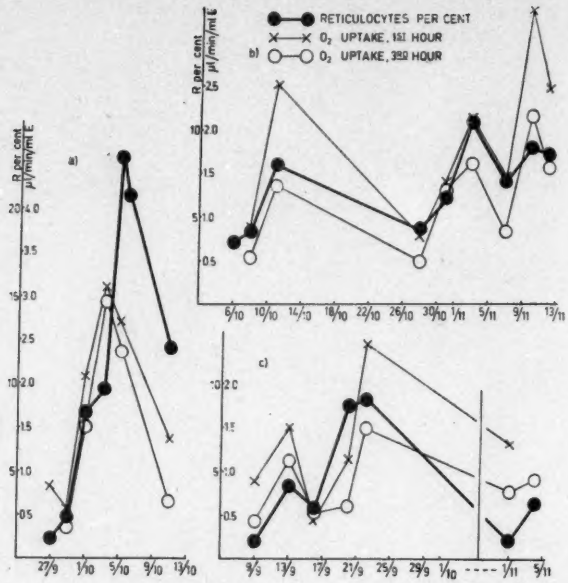


Fig. 2. Oxygen consumption of blood and percentage of reticulocytes. Abscissa: time, dates.

reticulocytes and blood oxygen consumption is quite clear. The dates at which the determinations are made represent the abscissa and the ordinates are reticulocyte percentage and oxygen uptake during the 1st and 3rd hours of incubation. It is obvious that a decline in percentage of reticulocytes immediately induces a reduction in the oxygen uptake of the blood. However, the oxygen consumption may decrease already *before* the reticulocytes have attained their peak value, although it never lags behind. Examples of earlier decline of oxygen consumption than of the reticulocytosis is seen in Fig. 2 a, 3 a, b and d. The explanation of this fact is probably very simple, as reticulocytes are counted cells with any amount of reticular substance. When the peak value of reticulocytosis is attained a great amount of cells are about to lose their substantia reticulofilamentosa and thus represent cells which are in their enzymatic constitution closer to the mature state than those with abundant staining material.

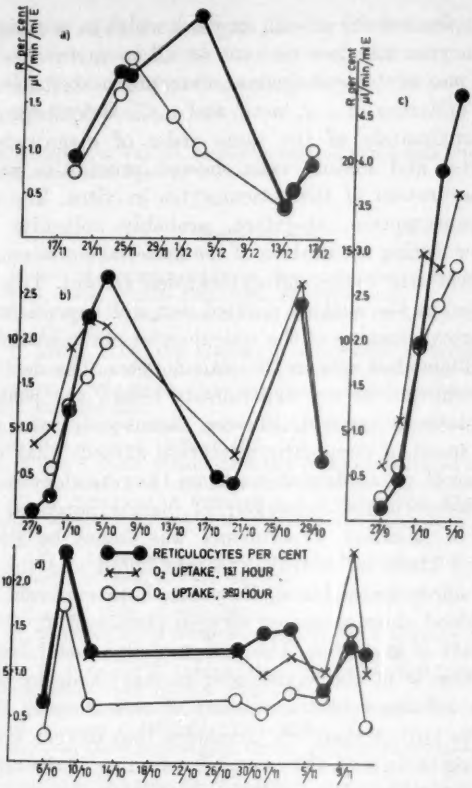


Fig. 3. Oxygen consumption of blood and percentage of reticulocytes.
Abscissa: time, dates.

Discussion.

The increased oxygen consumption of red cells manifested in connection with a reticulocytosis represents a very short episode in the life cycle of the red cell, and cannot, therefore, be taken as an indication of the mean age of the red cell population. This is in conformity with the findings of RUBINSTEIN, OTTOLENGHI and DENSTEDT (1956) concerning the decrease in the enzyme activity of reticulocytes in vitro. During an incubation of only 12 hours duration the authors were able to observe a notable

decrease in the activity of such enzymes which have a high activity in reticulocytes and low or none at all in mature cells as *e. g.* fumarase, succinic dehydrogenase, cytochrome oxidase and aconitase. The activities of *e. g.* lactic and malic dehydrogenase which were approximately of the same order of magnitude both in reticulocytes and mature cells showed practically no decrease during maturation of the reticulocytes in vitro. The decline in oxygen consumption, therefore, probably coincides with disappearance during maturation of the material containing enzymes of the citric acid cycle and cytochrome system. This maturing is, however, as the authors pointed out, not dependent upon the morphological ripening of the reticulocytes nor related to the contents of ribonucleic acid in the reticulocytes. The decline of oxygen consumption in my experiments before the peak value of reticulocytosis is reached, likewise corroborates the impression that the speed of enzymatic alteration exceeds that of the disappearance of stainable material from the reticulocytes. A similar relation between the behaviour of plasma potential and reticulocytes in bleeding experiments was found by PIHA (1956).

In sheep blood no reticulocytes are found and they are very scarce in sheep foetal blood. However, both maternal and foetal arterial blood show increased oxygen consumption at about the middle part of gestation. The maternal and foetal blood oxygen consumption is of the same order of magnitude in spite of the very big difference in the amount of reticulocytes (KARVONEN 1949). The author therefore concludes that oxygen consumption has nothing to do with the basophilic staining of the reticulocytes. The highest values of oxygen consumption in sheep maternal and foetal blood were of the same order as those found in my experiments on rabbit blood when the percentage of reticulocytes was about 10. Since the staining properties of the reticulocytes are very sensitive to various factors: liver extracts, salts, etc. (HEATH and DALAND 1931) it is possible that the (physico-)chemical constitution of sheep red cells or plasma is not favourable for staining of the reticulocytes and the discrepancy between sheep and rabbit red cell behaviour may be only apparent. On the other hand, the parallelism between oxygen consumption and percentage of reticulocytes in rabbit blood may also be coincidental, the speed of morphological ripening being approximately the same as the speed of enzymatic deterioration. The former can therefore mostly be used as an index for the latter.

The oxygen uptake of blood plasma is obviously too small in order to be determined with the necessary accuracy and probably too variable to be of any value in evaluation of the posthaemorhagic state of the animal.

Aided by grants from Valtion luonnontieteellinen toimikunta (State Commission for Sciences) and Sigrid Jusélius' Stiftelse.

References.

Brecher, G. and M. Schneiderman, A time-saving device for the counting of reticulocytes. *Amer. J. clin. Path.* 1950. *20.* 1079—1083.

Harrop, Y. A., The oxygen consumption of human erythrocytes. *Arch. intern. Med.* 1919. *23.* 745—7.

Heath, C. W. and G. A. Daland, Staining of reticulocytes by brilliant cresyl blue. Influence of solutions and substances. *Arch. intern. Med.* 1931. *48.* 133—145.

Hollingsworth, J. W., Erythrocyte glycolysis in hemolytic disease. *J. Lab. clin. Med.* 1955. *45.* 920—925.

Jalavisto, E., Bleeding anaemia and methaemoglobin reduction in dog erythrocytes. *Acta physiol. scand.* 1959. *46.* 252—256.

Jalavisto, E. and S. Piha, Plasma oxidation reduction potential and percentage of reticulocytes. *Ann. Med. exp. Biol. Fenn.* 1952. *30.* 361—372.

Jalavisto, E., A. Pitkänen, A. Selonen and L. Solanterä, The rate of methaemoglobin reduction in red cells of moderately bled rabbits. *Acta physiol. scand.* 1959. *46.* 257—272.

James, A. T., J. E. Lovelock and J. Webb, The biosynthesis of fatty acids by human red cells. *Biochem. J.* 1957. *66.* 60 P.

Karvonen, M. J., Oxygen consumption of sheep maternal and foetal blood. *Acta physiol. scand.* 1949. *17.* 267—275.

Komatsu, M., Enzymes in red blood cells. III. On the respiratory metabolism of reticulocytes. *Tohoku J. exp. Med.* 1956—57. *3.* 125—132.

Matthies, H., F. Jung and K. Onnen, Über den Stoffwechsel jugendlicher Blutzellen. *Dtsch. Gesundhwes.* 1953. *8.* 1562—1565.

Parsons, R. E. and W. Parsons, Observations on some processes of oxidation in bloodserum. *Biochem. J.* 1927. *21.* 1194—1205.

Piha, S., Studies on blood regeneration during progressive reticulocytosis due to repeated bleedings. *Ann. Acad. Sci. Fenn.* 1956. A. II. p. 37—38, 42.

Räihä, N., Hemolysis of human blood caused by oxygen and its prevention with vitamin E. *Acta Paediat.* 1955. *44.* 128—131.

Ramsey, R. and C. O. Warren, Jr., The rate of respiration in erythrocytes. *Quart. J. exp. Physiol.* 1930. *20.* 213—229.

Ramsey, R. and C. O. Warren, Jr., II. The rate in mature rabbit erythrocytes. *Quart. J. exp. Physiol.* 1933. *22.* 49—56.

Ramsey, R. and C. O. Warren, Jr., Oxygen consumption of rabbit red cells during lysis, with some observations on the oxygen consumption of plasma. *Quart. J. exp. Physiol.* 1934/35. *24.* 153—160.

Rubinstein, D., P. Ottolenghi and O. F. Denstedt, The metabolism of the erythrocyte XIII Enzyme activity in the reticulocyte. *Canad. J. Biochem. Physiol.* 1956. *34.* 222—235.

Selvoyn, J. G. and J. N. Dacie, Autohemolysis and other changes resulting from the incubation in vitro of red cells from patients with congenital hemolytic anemia. *Blood* 1954. *9.* 414—438.

Umbreit, W. W., R. H. Burris and J. F. Stauffer, Manometric techniques and related methods for the study of tissue metabolism. *Burgess Publ. Co. Minneapolis. Minn.* 1945.

Warburg, O., Zur Biologie der roten Blutzellen. *Hoppe-Seyl. Z. physiol. Chem.* 1909. *59.* 112—121.

Yamamoto, D., Quot. by Komatsu.

Acta physiol. scand. 1959. *46*. 252—256.

From the Institute of Physiology, University of Helsinki, Finland.

Bleeding Anemia and Methemoglobin Reduction in Dog Erythrocytes.

By

EEVA JALAVISTO.

Received 26 January 1959.

Abstract.

JALAVISTO, E. Bleeding anemia and methemoglobin reduction in dog erythrocytes. *Acta physiol. scand.* 1959. *46*. 252—256. — The rate of methemoglobin reduction in nitrite treated dog red cells were studied in 3 dogs before and after repeated bleedings. When the dogs were bled the methemoglobin reduction rate (MRR) increased to values twice or three times the initial, the maximum rate varying from 18 to 31 per cent per 1 hour. The duration and level of an elevated rate seemed to depend on the amount of blood removed. The long persistence (about 1 month) of an elevated MRR was interpreted as an indication of the dependence of the MRR on the age and life span of the red cell population.

The rate of methemoglobin reduction (MRR) in nitrite treated red cells is known to vary in different animal species (KIESE and WEIS 1943). It is high in rabbit and guinea pig red cells and low in dog and human erythrocytes both when glucose and lactate are used as substrates. A species difference exists likewise in the percentage of reticulocytes which is higher in rabbits and guinea pigs and lower in dogs and man. Since repeated bleedings enhance the rate of reduction in rabbit red cells (MATTHIES, JUNG and ONNEN 1953, JALAVISTO 1957, JALAVISTO *et al.* 1959) it seemed interesting to study the effect of bleeding dogs, which develop

usually a very moderate reticulocytosis in connection with bleeding anemia. The elevated rate of reduction persists for a rather long time, some 20 days in rabbits (JALAVISTO *et al.* 1959), but the time relationships are difficult to assess in rabbits since the sample taken for analysis is comparatively large and may stimulate the rate of erythropoiesis. In dogs a sample of the same size can hardly affect the bone marrow activity to any noticeable extent. Consequently the mere taking of a sample for analyses does not induce an elevated rate of methemoglobin reduction. The time relationships of the enhanced reduction rates are therefore easier to study in dogs.

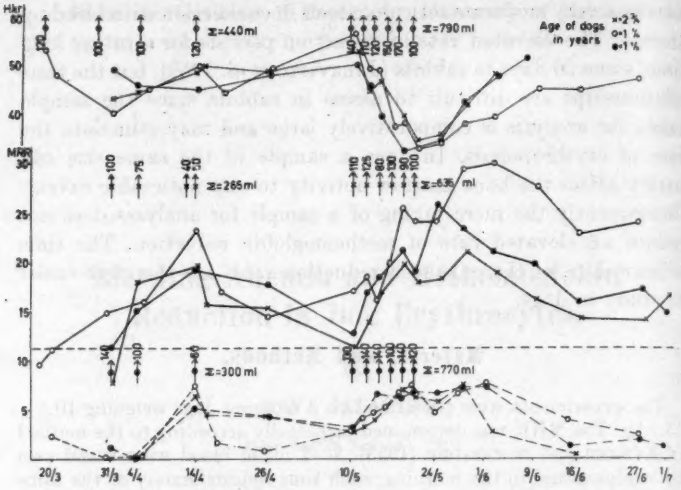
Material and Methods.

The experiments were performed on 3 mongrel dogs weighing 10.5—15.0 kg. The MRR was determined principally according to the method of KÜNZER and SCHNEIDER (1953). 2—4 ml of blood were withdrawn by venipuncture in the morning, each time approximately at the same hour. The samples were kept in icewater until diluted with 1 per cent sodium nitrite solution and incubated during $^1/_2$ hour at 37° C. The erythrocytes were separated from plasma and were washed 5 times with saline and suspended in a phosphate buffer solution adjusted to pH 7.4 to make 2 ml. Ten mg of glucose were added to this amount of suspension. A drop of the suspension was dissolved in distilled water and the content of methemoglobin was determined spectrophotometrically at the wavelengths 630 and 577 mμ according to the nomographic method of HUNTER (1951). After exactly 1 hour incubation in Warburg flasks at 37.1° C under shaking, a second sample was similarly analysed. The difference in methemoglobin concentration between the first and second sample was expressed as percentage of the initial concentration and was taken to represent the MRR. The methodical error in 26 double determinations was found to be $S = \sqrt{\frac{\Sigma \Delta^2}{2n}} = 0.91$ %/1 hour. In some experiments parallel determinations of MRR, after addition of 0.002M iodoacetic acid, were made.

The dogs were bled mostly every other day 3 to 6 times. 60—170 ml blood was removed at a time. The volume of packed cells was determined with a v. Allen's hematocrit.

Results.

The result is depicted in Fig. 1 in which the MRR %/1 hour and the haematocrit are plotted against time, *i. e.* dates at which the determinations were performed. The dogs were bled at days



Hematocrit (Hkr) and methemoglobin reduction rate (MRR) in nitrite treated dog red cells during two periods of bleeding.

Ordinate, upper curves: Hkr per cent,
lower curves: MRR per cent/1 hour.

Abscissa: time, dates. At arrows the dogs were bled.
The numbers indicate the amount of blood removed in ml.

Solid lines: MRR without inhibitor, glucose 0.5 %.

Broken lines: MRR inhibited with 0.002M iodoacetic acid, glucose 0.5 per cent.

shown by the arrows. The number on the arrow indicates the amount of blood taken in ml.

The dogs were subjected to two periods of bleedings — the first with only 3—4 venesections and the second with 6 bloodlettings during the $3^1/_2$ months time of observation.

The amplitude and duration of the increase in MRR seemed to be dependent upon the amount of blood removed. During the first bleedings when the blood removed represented 2.0—2.9 per cent of body weight, the maximum rate of MR varied between 18—23 per cent per hour. After about one month the original level was attained in the dogs from which 265—300 ml had been removed, whereas in the dog from which 440 ml (= 2.9 % body weight) was taken, the initial level of MRR was then not yet fully restituted. During the second period of blood letting when the amount of blood removed corresponded to 4.7—7.3 per cent

of body weight the maxima of MRR varied between 20—31 per cent per hour and after a month in none of the dogs had the rate of MR reached the original level. The highest rate was again found in the cells of the dog with the (absolutely, not relatively) largest blood loss, 790 ml (= 5.3 % body weight), and lowest in the cells of the dog with the smallest blood loss, 635 ml (= 4.7 % body weight).

The broken lines in the figure indicate the MRR when the cells have been incubated together with 0.002 M iodoacetic acid (and glucose). It may be noted that the residual reduction which persists in spite of the inhibitor is likewise speeded up by the bleedings. A similar increase in the MRR of iodoacetic acid poisoned cells was, however, found as well after injections of adrenaline in rabbits (JALAVISTO and SUNDBERG 1957).

Discussion.

The rate of methemoglobin reduction in dog erythrocytes is definitely slower than in rabbit red cells. The values in normal conditions before bleeding were approximately of the same magnitude, 9.5 per cent/hour, as mentioned in previous papers, *e. g.* 11.4 per cent of total pigment per hour as found by COX and WENDEL (1942). The effect of bleedings on the reduction rate, however, manifests itself as clearly in dog as in rabbit red cells.

The relation of the level of MRR to the amount of blood lost seems to indicate that the rate of reduction of methemoglobin is a function of red cell age. This will be treated more quantitatively in a subsequent paper (JALAVISTO and SOLANTERÄ 1959).

There seems not to be any big difference between the behaviour of dog and rabbit erythrocytes after bleedings as to the duration of the elevation of MRR. It is in both animals long lasting. The exact duration of an elevated rate has, however, not been assessed in either species. An initial drop in the level of MRR occurs both in dogs and rabbits soon after the blood lettings are stopped, but it takes about 20—30 days, sometimes even longer, before the initial level is regained. Whether the early decrease in the MRR indicates the existence of cells with short life span *i. e.* increased destruction of cells shortly after the bleedings remains unsettled. It would, however, very well fit in the present knowledge about life span of red cells after blood loss as studied *e. g.* by the isotope

C^{14} method (PIHA 1956). In these studies the C^{14} labelled globin remains stable during some 30 days in normal rabbits whereas after repeated bleedings there is no such plateau and a decrease in radio-activity of the globin fraction begins already after a few days. LINMAN and LONG (1958) have, furthermore, shown the production of cells with short life span by a lipid soluble heat-stable erythropoietin isolated from anemic plasma. Another possible interpretation would be that the blood loss has increased the rate of enzymatic maturation (or decay) of the cells. So far we know, there is at present no evidence whatsoever relating to this possibility.

Aided by grants from "Valtion luonnontieteellinen toimikunta" (State Commission for Sciences) and "Sigrid Jusélius' Stiftelse".

References.

COX, W. W., and W. B. WENDEL, The normal rate of methaemoglobin reduction in dogs. *J. biol. Chem.* 1942. *143.* 331—.

HUNTER, F. T., Quantitation of Mixtures of Haemoglobin Derivatives by Photoelectric Spectrophotometry. *Springfield, Ill.* 1951. pp. 226.

JALAVISTO, E., Effect of repeated bleedings on the rate of reduction of methaemoglobin in rabbit erythrocytes. *Acta physiol. scand.* 1957. *42.* Suppl. 145. 77—78.

JALAVISTO, E. and L. SOLANTERÄ, Methaemoglobin reduction in nitrite treated red cells as a function of cell age. *Acta physiol. scand.* 1959. *46.* 273—283.

JALAVISTO, E., A. PITKÄNEN, A. SELONEN and L. SOLANTERÄ, The rate of methaemoglobin reduction in red cells of moderately bled rabbits. *Acta physiol. scand.* 1959. *46.* 257—272.

JALAVISTO, E. and J. SUNDBERG, The effect of exposure to low pressure, of restraining and of injections of adrenaline on the rate of reduction of methaemoglobin in rabbit erythrocytes. *Ann. Med. exp. Biol. Fenn.* 1957. *35.* 250—257.

KIESE, M. and B. WEIS, Die Reduction des Hämoglobins in den Erythrocyten verschiedener Tiere. *Arch. exp. Path. Pharmak.* 1943. *202.* 494—501.

KÜNZER, W. and O. SCHNEIDER, Zur Aktivität der reduzierenden Fermentsysteme in den Erythrocyten junger Säuglinge. *Acta haemat.* 1953. *9.* 346—353.

LINMAN, J. W. and M. J. LONG, Erythrocyte osmotic fragility of rats receiving the thermostable plasma erythropoietic factor. *Blood* 1958. *13.* 226—238.

MATTHIES, H., F. JUNG and K. ONNEN, Über die Stoffwechsel jugendlicher Blutzellen. *Dtsch. Gesundhwes.* 1953. *8.* 1562—5.

PIHA, S., Studies on Blood Regeneration During Progressive Reticulocytosis Due to Repeated Bleedings. *Ann. Acad. Sci. Fenn.* 1956. Ser A. II. 80 pp. 99.

Acta physiol. scand. 1959. *46*. 257—272.

From the Institute of Physiology, University of Helsinki, Finland.

The Rate of Methemoglobin Reduction in Red Cells of Moderately Bled Rabbits.

By

E. JALAVISTO, A. PITKÄNEN, A. SELONEN and L. SOLANTERÄ.

Received 26 January 1959.

Abstract.

JALAVISTO, E., A. PITKÄNEN, A. SELONEN and L. SOLANTERÄ. The rate of methemoglobin reduction in red cells of moderately bled rabbits. *Acta physiol. scand.* 1959. *46*. 257—272. — Repeated bleedings were shown to enhance the methemoglobin reduction rate (MRR) of nitrite treated red cells incubated without substrate, with glucose at 7.4 and with lactate at pH 8. When the samples were incubated with adenosine prior to the treatment with nitrite the MRR was speeded up; the difference, however, between anemic and normal cells persisted. Regularly in anemic and sometimes in intact red cells pyruvic acid slowed the reduction rate down by a fairly constant amount. It was concluded that reduction through lactate cannot represent the main factor in the anemic increase of MRR. After bleedings when the reticulocytosis had subsided, an elevated MRR could still be observed. It was concluded that the anemic increase of MRR is due to an overall activation of metabolic processes in a population consisting of young red cells.

The reduction of methemoglobin in nitrite treated but otherwise intact red cells is known to be greatly enhanced after repeated bleedings (MATTHIES, ONNEN and JUNG 1953, MATTHIES 1956, JALAVISTO 1957). This increase was shown by MATTHIES to be linearly related to the percentage of reticulocytes and was supposed to be due to the altered enzymatic constitution of the reticulocytes. The rôle of the methemoglobin reductase isolated and studied especially by KIESE (1944) and KIESE, SCHNEIDER and WALLER (1957) was thought of as the most likely element involved.

If methemoglobinocytes are mixed with cells containing only hemoglobin the reduction rate in the former is increased. ("Mischeffekt" von MATTHIES, JUNG and SCHÄFER 1955.) The effect was shown to parallel the amount of lactic acid formed in the hemoglobin containing erythrocytes. Reticulocytes on the other hand did not possess this reduction enhancing effect, which was explained by their greater respiration and smaller lactic acid formation. Since PIHA (1958) has shown that the pyruvate content of red cells in repeatedly bled rabbits is increased, and pyruvate is known to inhibit the reduction of methemoglobin by lactate, this last-mentioned explanation would seem valid. However, PIHA has likewise shown (unpublished results) that a high lactic acid content in red cells with increased percentage of reticulocytes is to be found after repeated bleedings as well. Thus the whole matter seems to be quite confusing. Furthermore, if one considers the fact that the percentage of reticulocytes may be only moderately increased and yet the rate of methemoglobin reduction (MRR) is definitely enhanced the question becomes all the more complicated. It is simple to calculate, that even if the reticulocytes would reduce all methemoglobin they contain to hemoglobin during the incubation period (MRR = 100 %) it would not suffice to account for the observed increase in the mean rate of methemoglobin reduction (JALAVISTO 1957). Obviously the question deserves further study.

Material and Methods.

The material comprises orienting experiments from an early period (spring 1955) and main experiments performed between April 1957 and November 1958. The aim of the orienting experiments was to study the degree and duration of reticulocytosis in relation to the increase in the rate of methemoglobin reduction. The rabbits were in

these experiments bled at varying intervals and samples were taken at and after the bleedings.

The samples were taken from the marginal ear vein and in the orienting experiments put immediately in an ice cooled water bath. The reticulocytes were counted using Miller's ocular micrometer whereat 20 squares were counted, which gives approximately the same accuracy as if 1,000 erythrocytes would have been counted. The samples were stained with brilliant cresyl blue. Determination of the hematocrit value and/or the number of erythrocytes were likewise performed. Irregularities in the MRR led to search for an explanation and it was discovered that restraining of the rabbits may lead to increased MRR probably through mobilization of adrenaline (JALAVISTO and SUNDBERG 1957). The experimental procedure was therefore altered in the main part of experiments. In order to avoid the effect of restraining, the animals were treated as gently as possible and the blood was taken by heart puncture, the rabbit lying on its back in an animal holder in a state known as "animal hypnose". To control variations due to occasional differences in the interval before the blood sample was ready for determination of the MRR, the blood samples were always taken from a pair of rabbits: one repeatedly bled and another intact rabbit which served as a control. The control and bled rabbits and the samples were treated throughout in exactly the same manner. Coagulation of the blood was prevented with a drop of heparin (5,000 I. U. in 1 ml) introduced into the syringe and the flasks in which the blood was collected. The reticulocyte percentage was determined as in the orienting experiments. The percentage of reticulocytes varied between 0.8—3.7 % in the controls and between 4.3—11.7 % in the bled rabbits; in addition red cells were counted and the hemoglobin contents were determined colorimetrically as alkaline hematin.

The determination of the MRR was performed as follows. Two ml of blood (total amount taken was usually 20—25 ml per rabbit) were placed in two centrifuge tubes, filled with 1 per cent solution of sodium nitrite and incubated for 30 min in a water bath of 37° C. The samples were washed five times with saline (or phosphate buffer) in order to remove all nitrite (three or four washings are not sufficient). The last washing was made with isotonic phosphate buffer of pH 7.4 (or in general with the pH to be maintained during incubation). The washed cells were in the orienting experiments suspended in a phosphate buffer solution to make approximately 2 ml, which were pipetted in Warburg flasks to which 10 mg or 0.1 ml of a 10 % solution of glucose was added. In the final experiments the cells were suspended in 5 ml of phosphate buffer and 2 ml of the suspension were placed in duplo into three (a—c) Warburg flasks: a) with no supplement, b) containing 0.1 ml of a 10 % solution of glucose and c) containing 0.15 ml of a 1.72 per cent isotonic sodium lactate solution. The flasks were incubated in a water bath of 37° C under gentle shaking during 1 hour. The methemoglobin percentage was determined in the preliminary and final experiments by spectrophotometry at wave lengths 577 mμ and 630 mμ according to HUNTER, both before and after

incubation. The MRR was expressed as the decrease in methemoglobin contents in per cents of the initial value. In some experiments the blood was incubated during one hour in 37° C with an 0.034 per cent solution of adenosine before the blood samples were treated with nitrite.

The rest of the blood was stored at + 5° C for about 24 hours. Thereafter the incubation with adenosine and the determination of MRR were performed as described above.

In some instances the lactic acid contents of blood samples taken after the incubation in Warburg flasks were determined according to the method of BARKER and SUMMERSON (1941) in which the lactic acid is converted to acetaldehyde through the action of concentrated sulphuric acid. Acetaldehyde gives in presence of copper ions a purple colour with p-hydroxydiphenyl and may thus be determined by colorimetry. The standard calibration curve was made by diluting the same isotonic 1.72 % sodium lactate stock solution that was used in the incubation experiments as substrate.

The rabbits were kept on a stock diet consisting of hay, oats and barlow, supplemented once a week with fir-needles. They were bled at irregular intervals, mostly every second day.

Results.

Preliminary experiments. The MRR when glucose was used as substrate varied in 100 blood samples between 39 and 85 per cent/hour. In only 4 instances the MRR exceeded 80 per cent. The range of reticulocyte percentage was 0.5—24.0, the mean being 8.7 %. If the rabbits had not been bled the MRR was about 50 per cent and the reticulocyte percentage 1.5—2.0 %, but the correlation was otherwise very poor. The correlation coefficient calculated from the entire material (100 samples) was $r = + 0.20 \pm 0.097$, thus only bordering significance. It may be noted that when a low reticulocyte percentage is found in connection with a high rate of reduction, the rabbit has usually been bled, but the reticulocytosis has already subsided. A relatively long persistence of an elevated MRR is seen almost invariably. Some examples are given in Fig. 1 in which MRR, hematocrit and reticulocyte values of 5 rabbits are represented. An evaluation of the duration of the elevated MRR after bleeding is rather difficult because the taking of a blood sample for analysis acts by itself as a regenerative stimulus and may enhance the MRR. The determinations of MRR cannot, therefore, be repeated as often as would be desirable. The general impression when examining the curves in Fig. 1 is, that the MRR remains elevated some

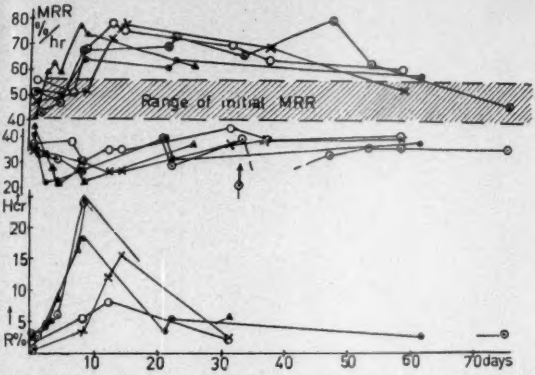


Fig. 1. Rate of methemoglobin reduction (MRR) %/hour in nitrite treated red cells of 5 rabbits. In the middle part are shown the hematocrit values (Hcr) and at the bottom the percentage of reticulocytes (R%). Abscissa: time in days after beginning of bleedings.

20 days and occasionally even longer. The parallelism between reticulocytosis and increase in MRR is quite obvious at the beginning of the bleedings and it is only the later development which breaks the correlation.

Main experiments. The results appear in Tables I—IV. In Table I the MRR in cells incubated without added substrate are shown. A reduction of methemoglobin is taking place both in control and bled rabbit cells. The rate is so slow compared to methodical or other variations that a significant difference between normal and bled rabbits is only seen if the differences between each pair of normal and bled rabbit is calculated (column 5). The difference is seen as well in experiments with suspension media of pH 6.8 in which the MRR is suppressed (bottom of Table I).

From Table I, the effect of incubation with adenosine on the reduction rate when no substrate is added can likewise be seen. The rate is increased on an average with some 50 per cent in the fresh samples and in stored samples the rate is more than doubled. In the latter case the increase is statistically significant.

If it is assumed that the increase is due to the reticulocytes reducing methemoglobin at a higher rate, one may calculate the minimum percentage of reticulocytes needed to bring about an increase in the mean MRR of the magnitude found experimentally. The MRR can maximally be 100 %. If the percentage

Table I.

MRR %/hour, without added substrate, pH 7.4.

Sample	Controls R = 2.4 ± 04. per cent		Bled R = 7.7 ± 1.1 per cent		Difference		
	MRR %/hour	n	MRR %/hour	n	in MRR %/hour	t	P
Fresh blood cells	1. 9.83 ± 1.23	8	5. 13.27 ± 1.92 R calc = 6.1 %	8	5—1: + 4.32 ± 1.30***	3.22	< 0.01
Stored for 24 hours	2. 6.52 ± 1.36	7	6. 10.14 ± 1.06 R calc = 6.8 %	7	6—2: + 3.62 ± 1.16***	3,12	< 0.01
Fresh, adenosine pretreated .	3. 14.4 ± 2.97	9	7. 18.25 ± 2.82 R calc = 6.8 %	9	7—3: + 3.86 ± 2.65	1.51	> 0.1
Stored adenosine pretreated.	4. 14.3 ± 3.00	7	8. 19.73 ± 3.50 R calc = 8.5 %	7	8—4: + 5.35 ± 2.32*	2.31	< 0.05
Difference	2—1: − 3.31 ± 1.85[1]		6—5: − 3.13 ± 2.19		* significant, P < 0.05		
Difference	3—1: + 4.57 ± 3.2		7—5: + 4.98 ± 3.4		** » P ~ 0.02		
Difference	4—2: + 7.7 ± 3.29*		8—6: + 9.59 ± 3.62**		*** » P < 0.01		
Fresh blood cells pH 6.8	9. 5.66 ± 1.8	5	10. 10.2 ± 2.0	5	10—9: +4.52 ± 1.35	3.34	0.01

[1] That the mean difference is not significant depends mainly on the great interindividual variations, not on variation in the direction of the change. Thus in groups 1 and 2 out of 7 values of MRR 6 were smaller after storage, 1 was equal to and none was greater than the corresponding value of the fresh sample.

corresponding value of the fresh sample.

Table II.

Incubation with glucose 0.5 % as substrate, pH 7.4.

	Controls (R = 2.4 ± 0.4 %)		Bled (R = 7.7 ± 1.1 %)		Difference		
	MRR %/hour	n	MRR %/hour	n	MRR %/hour	t	P
Fresh blood cells	1. 51.0 ± 2.95	9	5. 64.7 ± 5.0 R calc = 29.4 %	9	5—1: + 15.2 ± 4.9	3.1	< 0.02
Stored for 24 hours	2. 31.2 ± 2.5	6	6. 45.4 ± 5.2 R calc = 22.5 %	6	6—2: + 14.2 ± 4.8	2.95	< 0.05
Fresh, *adenosine* pretreated cells	3. 64.5 ± 3.1	9	7. 79.0 ± 3.5 R calc = 42.5 %	9	7—3: + 14.5 ± 4.8	3.03	< 0.02
Stored, *adenosine* pretreated cells	4. 54.3 ± 5.5	8	8. 73.1 ± 3.7 R calc = 42.5 %	8	8—4: + 18.8 ± 5.3	3.56	< 0.01
Difference in MRR %/hour....	2—1: − 19.8 ± 3.85		t = 5.15	P < 0.001	6—5: − 19.3 ± 7.21	2.67	< 0.02
Difference in MRR %/hour....	3—1: + 13.5 ± 4.26		t = 3.2	P < 0.01	7—5: + 14.3 ± 6.10	2.3	< 0.05
Difference in MRR %/hour....	4—2: + 23.1 ± 6.05		t = 3.8	P < 0.01	8—6: + 27.7 ± 6.46	4.3	< 0.001

of reticulocytes in the control experiments is 2.4 and the mean MRR 9.8/hour then mature cells would reduce 7.6 per cent/hour and there should be at least 6.1 per cent reticulocytes in order to yield a mean reduction rate of 13.3 as in the anaemic rabbit cells. The calculated minimum percentage (R calc.) 6.1—8.5 (adenosine pretreated) corresponds to the reticulocyte percentage actually found.

In Table II the results with added glucose are shown. The reduction rate is now much increased and evidently more so in the red cells from bled rabbits. Again it may be noted that the difference is significant both in fresh and stored samples. The storage has resulted in a marked decrease in MRR. The effect of adenosine pretreatment is striking: the MRR in stored samples is not only restored to the former level but even increased beyond the original rate of reduction. The increase is particularly high in the red cells of bled rabbits. Again we may calculate the minimum percentage of reticulocytes needed to yield the mean reduction rate found in the experiments if supposed that the MRR enhancing effect of bleedings would be dependent upon higher rate of reduction in the reticulocytes. The values (R calc.) are shown in the table. It is obvious that the percentages (R calc.) are much too high and consequently a higher rate in reticulocytes cannot explain the increase in MRR after bleeding. Either there must be cells not staining as reticulocytes but reducing at a higher rate than ordinary mature erythrocytes or there must be interaction between the cells as shown by MATTHIES *et al.* in the "Mixing Effect".

There is very little difference between the MRR in control and bled rabbit cells, when lactate is used as substrate (Table III) — a fact shown by MATTHIES *et al.* The rate is to begin with much higher than with glucose: actually the same degree of reduction is reached already in half an hour. In every group the MRR is numerically slightly greater in bled rabbit cells than in controls, but the difference is not statistically significant. The rate is not reduced during storage and is not essentially increased with adenosine pretreatment.

The lack of a significant difference between controls and bled rabbit blood cells may be due to the fact that reduction of methemoglobin proceeds with lactate at such a high rate even in controls that a slight increase may be masked by methodical inaccuracy resulting of the necessarily short time of observation,

Table III.

Incubation with 0.013 M sodium lactate pH 7.4.

Sample	Controls		n	Bled		n	Difference bled—control		t	P
	MRR %/hour			MRR %/hour			in MRR %/hour			
	½ hour	1 hour		½ hour	1 hour		½ hour	1 hour		
Fresh cells	61.9 ±	84.3 ± 3.4	5	68.6 ± R calc = 19.9 %	90.0 ± 2.0	5	+ 6.2	+ 5.7	2	—
Stored for 24 hours	57.4 ± 1.8	81.1 ± 3.6	5	61.8 ± 3.1 R calc = 12.4 %	82.6 ± 2.6	5	+ 4.4	+ 1.5	2	—
Fresh, *adenosine* pretreated cells	57.8 ± 4.1	81.5 ± 2.9	6	69.1 ± 3.8 R calc = 28.5 %	90.1 ± 1.7	6	+ 11.2 ± 6	+ 8.6 ± 5.8	2	—
Stored, *adenosine* pretreated cells	57.1 ± 2.6	82.9 ± 2.8	7	63.6 ± 2.7 R calc = 17.1 %	87.5 ± 1.8	5	+ 6.5 ± 5.0	+ 4.6 ± 3.7	2	—

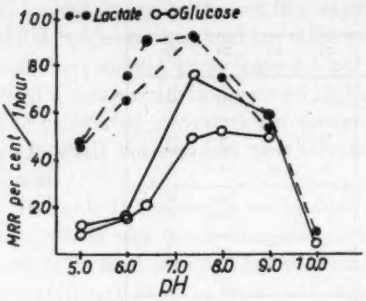


Fig. 2. Dependence of MRR on pH with lactate 0.013 M (broken lines) and glucose 0.5 per cent (solid lines) as substrates.

viz. $^1/_2$ hour. Since, however, it would be quite important to know whether there is a real difference between control and bled rabbit red cell MRR or not, it seemed necessary to find out conditions which would slow down the rate of reduction by lactate. The MRR was examined as a function of different hydrogen ion concentrations (Fig. 2). It could be noted that in the alkaline direction the reduction rate falls off steeper than at the acid range of pH. The curves of MRR with lactate and glucose, therefore, run closer to each other at the pH > 7.4. The pH 8.0—8.2 were chosen for incubation of the methemoglobin containing cells. The experimental procedure was altered in so far that the "control" values were taken from the same anemic rabbit blood. The bottom layer of red cells was used as control against the top layer rich in reticulocytes. The blood was centrifuged in narrow tubes, the plasma was discarded and the cells from two such tubes put together and centrifuged anew during 1 hour at a speed of 4,500 rpm. The top and bottom layers were taken apart and treated as two separate samples using the method described previously, except that the phosphate buffer was adjusted to pH 8.0 and 8.2. The results appear in Fig. 3.

It is noted that when the reticulocyte percentage is 2.0 % and the reduction rate is low there is no difference between top and bottom layer cells, but otherwise both in glucose and lactate the top layer cells reduce at a higher rate than the bottom layer cells. In the lactate series there was one exception but the difference is nevertheless quite significant: the mean difference is $+ 5.8 \pm 1.92$, and in the glucose series $+ 8.4 \pm 2.0$, respectively.

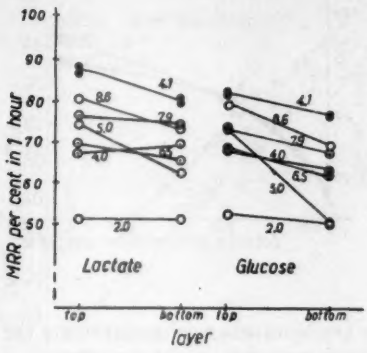


Fig. 3. MRR in bottom and top layer cells in centrifuged samples. The cells are incubated with lactate (0.013 M) and glucose (0.5 %). Numbers above the lines indicate percentage of reticulocytes in the original blood sample.

Inhibition of lactate reduction by added pyruvate.

The formation of lactic acid in the cells during incubation might be of importance for the increased MRR as it is for the "Mischeffekt" (mixing effect) of MATTHIES, JUNG and SCHÄFER (1955). The reduction by lactate and glucose as substrates are known to be additive and increasing amounts of lactate continue to enhance the MRR up to very high concentrations as may be seen from Fig. 4 which gives the MRR at various concentrations of added lactate.

The rôle of lactate can, however, only be estimated with help of experiments on inhibition of lactate reduction with pyruvate. In experiments with 0.013 M sodium lactate an addition of 0.027 M sodium pyruvate was found to depress the MRR approximately to the level found without added substrate. This concentration of pyruvate was, therefore, used in the following experiments.

When the MRR was determined with glucose as substrate with and without pyruvate at pH 7.4 a small reduction occurred regularly in the anemic and often even in the control samples. The reduction induced by addition of pyruvate amounted to 10.0 ± 1.03 per cent. This difference was, however, not related to the increase in the MRR. When the MRR with glucose is plotted against MRR with glucose plus pyruvate the values lie approximately on a straight line as seen from Fig. 5.

If the incubation was performed with a phosphate buffer of pH 6.8 no difference could be seen although in this pH range

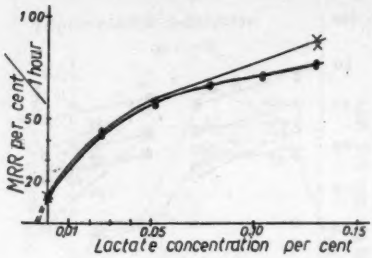


Fig. 4. Dependence of MRR on concentration of added lactate.

when lactate is used as substrate the MRR is only slightly reduced from its value at pH 7.4 (Fig. 2).

It is concluded that reduction through lactate may be one factor in anemic increase of MRR but it does not represent the main mechanism responsible for the increase. In acid range of pH it is not operative.

Interaction between methemoglobin reducing cells.

The rabbits were bled only moderately and the resulting increase in the percentage of reticulocytes was not great (mean 7.7 per cent). It is quite obvious (and was shown by calculations, Tables II and III) that under these conditions reticulocytes at however high rate they may reduce methemoglobin cannot account for the entire effect of bleeding on the MRR — at least not in experiments with added substrates. The long persistence of an elevated rate after subsidence of the reticulocytosis suggests that young red cells which assumably continue to glycolyse at a high rate (HOLLINGSWORTH 1956) reduce methemoglobin at a high rate too. Whether the amount of such young red cells is sufficient to account for the found difference in MRR can be estimated if the rate of blood regeneration in the post-bleeding period is known. This question will be considered in a separate paper (JALAVISTO and SOLANTERÄ 1959). However, the possibility to treat this matter quantitatively rests upon the assumption that the MRR is determined solely by the metabolic activity of the methemoglobin reducing cells themselves and is not influenced by diffusible metabolites formed in neighbouring cells as supposed to occur in the "Mischeffekt" of MATTHIES, JUNG and SCHÄFER (1955). To have this question settled the MRR was determined in samples

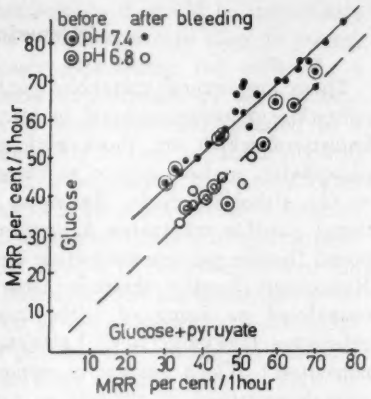


Fig. 5. MRR in control and bled rabbit red cells incubated with and without pyruvate.

after mixing cells which reduce at a slow rate with cells that reduce at a high rate. The result appears in Table IV and is quite clear cut: The reduction rate of the mixture corresponds exactly to the mean of the reduction rates of the original cell samples or is slightly below the calculated mean. Thus no effect of a diffusible substrate speeding up the reduction of slowly reducing cells can be detected in these experiments.

Table IV.

MRR of red cell suspensions obtained by mixing cells with high and low MRR, respectively, in varying proportions.

Rabbits	MRR, % /hour	Mixture	MRR, % /hour	Mixture	MRR, % /hour
1	83.4	1 part	73.0	1 part	69.4
2	63.0	1 part		2 parts	
		Calculated means	73.2		69.8
3	69.6	1 part	61.2	1 part	58.3
4	56.8	1 part		2 parts	
		Calculated means	63.2		61.1

Discussion.

There are several metabolic pathways possibly concerned in reduction of methemoglobin in nitrite treated red cells the best known of which are those with glucose (or glyceraldehyde-3-phosphate), and/or lactate as substrates and which are linked to the glycolytic cycle. Malonates and formaldehyde are additional possible substrates. A methemoglobin reductase, an interposed flavine enzyme-nucleotide system is furthermore required. Reduction directly through ascorbic acid or glutathione is considered as being of little importance. (KIESE (1947), for references GRANICK (1949) LEMBERG and LEGGE (1949) may be consulted.) Which system is responsible for the increased rate after hemorrhage is difficult to assess. The experiments with inhibition of reduction through lactate seemed to indicate that increased production of lactate cannot fully explain the phenomenon. The earlier experiments with iodoacetic acid inhibition which suppresses almost entirely the reduction of methemoglobin with glucose as substrate did not level out the difference between anaemic and normal cells in bleeding experiments with dogs (JALAVISTO 1959). An increased rate in anemic cells persisted also after incubation with adenosine which is known to regenerate the cells content of adenosine triphosphate. Likewise in samples after 24 hours storage which nearly depletes the nondialyzable endogeneous substrate of the cells, the difference is not abolished. The conclusion is, therefore, near at hand that no single factor can account for the whole effect and that the increased rate simply refects an increased overall metabolic activity of young red cells.

The poor correlation between the percentage of reticulocytes and the rate of methemoglobin reduction rises the question whether the reticulocytes are at all concerned with the increased reduction rate. It is well known that respiration prevails in reticulocytes, whereas in morphologically mature cells glycolysis is the energy yielding metabolic process. The respiratory metabolism represents a very short phase in the life cycle of the erythrocyte but during that phase accumulation of pyruvic acid takes place in the red cells as shown by the close parallelism between pyruvic acid contents and percentage of reticulocytes (PIHA 1958) during the progressive phase of reticulocytosis. Since pyruvic acid partly

inhibits the reduction of methemoglobin it would be more likely that reticulocytes would reduce at a slower rate than do young, morphologically mature intensely glycolysing red cells: As a matter of fact in the preliminary experiments we noticed that high percentages of reticulocytes sometimes corresponded to a remarkably low MRR. Since the newly formed metabolically still active cells very soon after bleeding outnumber the reticulocytes it might be possible that the enhanced rate is not directly connected with the presence of reticulocytes but with the rate of erythropoiesis. This again is reflected in the number of reticulocytes and the age structure of the red cell population. That the latter fact is the most important in this connection as will be shown in a subsequent paper. On the other hand it is possible that during very intense erythropoiesis cells defective in their enzyme systems may be formed and consequently fail to reduce methemoglobin at a high rate.

Summary.

1. The rate of methemoglobin reduction (MRR) in nitrite treated washed red cells of intact and moderately bled rabbits was investigated.

2. Repeated bleedings were shown to enhance the MRR of cells incubated without substrate and with glucose at pH 7.4 and at pH 8 also with lactate as substrate.

3. The difference between anemic and normal red cells was not abolished through storage at + 5° C for 24 hours which causes a marked slowing of the MRR. When the samples were incubated with adenosine prior to the treatment with nitrite the MRR was speeded up both in fresh and in stored samples. In the latter case the initial rate was thereby more than restored. The difference between anemic and normal cells persisted after incubation with adenosine. Regularly in anemic and sometimes in intact red cells pyruvic acid slowed the reduction rate down by a fairly constant amount. It was concluded that reduction through lactate cannot represent the main factor in the anemic increase of MRR.

4. After bleeding when the reticulocytosis had subsided an elevated MRR was still observable. The duration of this elevation could not be exactly determined but a rough estimate gives the value of some 20 days. The anemic increase of MRR probably

reflects an overall activation of metabolic processes in a population consisting of young red cells.

Aided by grants from Valtion luonnontieteellinen toimikunta (State Commission for Sciences) and "Sigrid Jusélius' Stiftelse".

References.

GRANICK, S., The chemistry and functioning of the mammalian erythrocyte *Blood* 1949. *4.* 404—440.

HOLLINGSWORTH, J. W., Erythrocyte glycolysis in hemolytic disease. *J. Lab. clin. Med.* 1955. *45.* 920—925.

HUNTER, F. T., Quantitation of Mixtures of Haemoglobin Derivatives by Photoelectric Spectrophotometry. *Springfield,* Ill. 1951. pp. 226.

JALAVISTO, E., Effect of repeated bleedings on the rate of reduction of methaemoglobin in rabbit erythrocytes. *Acta physiol. scand.* 1957. *42.* Suppl. 145. 77—78.

JALAVISTO, E., Bleeding anemia and methemoglobin reduction in dog erythrocytes. *Acta physiol. scand.* 1959. *46.* 252—256.

JALAVISTO, E. and L. SOLANTERÄ, Methemoglobin reduction in nitrite treated red cells as a function of cell age. *Acta physiol. scand.* 1959. *46.* 273—283.

JALAVISTO, E. and J. SUNDBERG, The effect of exposure to low pressure, of restraining and of injections of adrenaline on the rate of reduction of methaemoglobin in rabbit erythrocytes. *Ann. Med. exper. Biol. Fenn.* 1957. *35.* 250—257.

KIESE, M., Die Reduktion des Hämiglobins. *Biochem. Z.* 1944. *316.* 264—294.

KIESE, M., C. SCHNEIDER and H.-D. WALLER, Hämiglobinreductase. *Arch. exp. Path. Pharmak.* 1957. 231. 158—169.

KIESE, M. and W. SCHWARZKOPF, Die Reduktion des Hämiglobins. III Mitt. Reduktion des Hämiglobins und Stoffwechsel in roten Zellen. *Arch. exp. Path. Pharmak.* 1947. *204.* 267—287.

KÜNZER, W. and D. SCHNEIDER, Zur Aktivität der reduzierenden Fermentsysteme in den Erythrocyten junger Säuglinge. *Acta haemat.* (Basel) 1953. *9.* 346—353.

LEMBERG, R. and J. W. LEGGE, Hematin Compounds and Bile Pigments. *Interscience Publ. Inc. New York* 1949.

MATTHIES, H., Methämoglobin Rückbildung in Reticulocyten. *Arch. exp. Path. Pharmak.* 1956. *229.* 331—337.

MATTHIES, H., F. JUNG and K. ONNEN, Über die Stoffwechsel jugendlicher Blutzellen. *Dtsch. GesundhWes.* 1953. *8.* 1562—5.

MATTHIES, H., F. JUNG and R. SCHÄFER, Über Stoffwechselleistungen von roten Blutkörperchen. III. Zur Aufklärung des sogenannten Mischeffektes bei der Methämoglobinrückbildung. *Arch. exp. Path. Pharmak.* 1955. *225.* 352—358.

PIHA, R. S., Behaviour of pyruvic acid in plasma and red cells in rabbits bled repeatedly. *Acta physiol. scand.* 1958. *44.* Suppl. 151. pp. 43.

Acta physiol. scand. 1959. *46*. 273—283.

From the Institute of Physiology, University of Helsinki, Finland.

Methemoglobin Reduction Rate of Nitrite Treated Red Cells as a Function of Cell Age.

By

EEVA JALAVISTO and L. SOLANTERÄ.

Received 26 January 1959.

Abstract.

JALAVISTO, E. and L. SOLANTERÄ. Methemoglobin reduction rate of nitrite treated red cells as a function of cell age. *Acta physiol. scand.* 1959. *46*. 273—283. — The influence of cell age on the rate of reduction of methemoglobin (MRR) in nitrite treated red cells has been investigated. Starting from the assumption that the decrease of MRR with cell age proceeds logarithmically from 100 per cent at the second day to zero on the 160th or 180th day, the mean MRR was calculated for different cell ages. The agreement of the predicted and found values was good. The same logarithmically decreasing age decay curve could be used for calculation of the MRR both in rabbits and in dog and human erythrocytes if the maximum reduction rate is assumed to be 100 per cent/1 hour in rabbit and 35 per cent/1 hour in dog and human erythrocytes.

An enhanced rate of methemoglobin reduction (MRR) in nitrite treated red cells due to blood loss persists after the subsidence of reticulocytosis (JALAVISTO 1959, JALAVISTO *et al.* 1959). The increased MRR, therefore, cannot be due to the presence of a greater number of reticulocytes. It was assumed that the MRR may be considered as an indication of mean cell age, *i. e.* of the enzymatic youth of the red cell.

This paper is an attempt to treat the matter quantitatively and the data are essentially taken from the experiments published in the papers mentioned above. In this connection it is necessary to pay attention to the methods of estimation of the age structure of a red cell population after bleedings and the so called "mean cell life" since inadequacy and inaccuracies in these units necessarily affect the result of the intended quantitative comparison.

Methods.

The method of determination of the methemoglobin reduction rate performed essentially according to KÜNZER and SCHNEIDER (1953) has been described in a previous paper (JALAVISTO *et al.* 1959). Only those experiments in which glucose was used as substrate during incubation (1 hour at 37° C) were considered. In samples taken before and after incubation the percentage of methemoglobin was determined spectrophotometrically according to HUNTER (1951). The difference expressed as per cent of the initial value, was taken to represent the MRR (per cent/1 hour).

The red cells were counted in the usual way. The hemoglobin was determined with an EEL colorimeter in an alkaline solution. The percentage of reticulocytes related to 1,000 red cells was counted after staining with brilliant cresyl blue. The blood samples were taken either from exteriorized carotid loops or by heart puncture in rabbits or by venipuncture in dogs.

The erythrocyte count and hemoglobin percentage in samples taken after various intervals from blood letting were used for calculation of the rate of erythropoiesis and hemoglobin formation. For this purpose a formula proposed by PIHA (1956) was employed. In the formula estimated values for blood volume and for daily destruction of red cells are used. In rabbits the blood volume was calculated by assuming it to be 6—6.5 per cent, in dogs 7 per cent of the body weight. As daily destruction rate was taken 2 per cent in rabbits and 0.83 per cent in dogs, which would correspond to an average cell life of 50 days in rabbits and 120 days in dogs, respectively. The former figure corresponds to an average life span value obtained by PIHA (1956) when ^{14}C has been used for labelling the red cell globin. The formula gives accurate values only on condition that the blood volume remains a constant fraction of body weight, that there is no extra hemolyses of cells and that there is no alteration in the life span of red cells due to the experimentally induced conditions, and, of course, that the measured volume of blood removed gives the true blood loss, *i. e.* that there is no loss in subcutaneous tissue or elsewhere where it cannot be measured. The formula is as follows:

$$B = E'' - E' (1 - v'/V)/n + E' (1 - v'/V)/L$$

where:

B = rate of red cell production or hemoglobin formation.
E'' = number of erythrocytes per mm³ (in millions) or hemoglobin g/100 ml blood at nth day after first sample.
E' = initial erythrocyte count or hemoglobin percentage.
n = number of days between E'' and E'.
v' = volume of blood removed.
V = blood volume.
L = life span of red cells.

Inaccuracies of the blood count and of hemoglobin determinations affect the calculated mean red cell age and hemoglobin production. The determination of hemoglobin is usually considered as more accurate than the red cell count. Therefore, *e. g.* ALLISON and BURN (1955) relate the enzyme activity of blood to a unit quantity of hemoglobin instead of unit number of red cells. If conditions are stable this may be justifiable, but in experiments with bleeding anemia the hemoglobin contents of red cells may vary independently of cell production. When discrepancies between hemoglobin formation and red cell production (both expressed as per cent of actual contents) are found a comparison with the percentage of reticulocytes usually shows that the values for hemoglobin formation are misleading if they are taken to represent the rate of erythropoiesis. The not quite infrequent occurrence of negative values both for red cell production and hemoglobin formation suggests that the values must be considered as fairly rough approximations.

The mean cell life may be defined according to DORNHORST (1951) as follows:

total percentage of red cells (100 per cent)/percentage of red cells destroyed per day.

During stable conditions the daily destruction of red cells is equivalent with daily production of cells, but after a blood loss this no longer holds true. Since in our studies the rate of cell destruction could not be measured, the mean cell age could not be calculated either. However, if the rate of production of cells (per cent) is put in the formula instead of the destruction rate, one gets a measure of how many days it would have taken to form the present cell population if assumed that the rate would have been constant during the necessary number of days. If there is a sudden and temporary increase of erythropoiesis, such a measure is, of course, quite illusory. In lack of a better one, it is nevertheless used as representative of "mean cell age".

Results.

When the mean red cell production during a period preceding the blood lettings were calculated the values in 8 rabbits varied between 0.04—0.13 mill./mm³. In the post-bleeding period the rate remains for some time slightly elevated, being after 2—3 weeks 0.11—0.27 mill/mm³ (Table I). Expressed as percentage

Table I.

Mean daily production of red cells, and MRR before and after a period of bleedings.

Rabbit	Days preceding bleedings	Prehemorrhagic mean daily production of red cells		MRR %/1 hour	Days after last bleeding*	Posthemorrhagic mean daily production of red cells		MRR %/1 hour
		mill. E/mm³	per cent			mill. E/mm³	per cent	
239	2	0.13	2.1	41	—	—	—	—
R.	2	0.07	1.8	51	18—35	0.11	2.2	58.5
Pa.	2	0.07	1.5	53	0—14	0.23	4.2	80
II.	3	0.12	2.4	46	0—18	0.12	2.15	45.5
0	4	0.09	1.8	63.5	0—21	0.14	2.8	73
Ru.	12	0.08	2.1	52	0—25	0.15	2.4	68
J.	8	0.09	1.7	56	17—23	0.16	2.5	64
Pi.	8	0.04	0.75	47	16—22	0.27	4.5	69
Means		0.09	1.8	51.2		0.17	3.0	65.4

* Period to which the average production rate relates.

of the actual blood count the pre-bleeding daily production was on an average 1.8 per cent and the post-bleeding formation 3.0 per cent, respectively. It may be noted that the pre-bleeding percentage obviously corresponds to the normal percentage of reticulocytes in rabbits.

The rate of reduction of methemoglobin (MRR) is low, 51.2 per cent/hour before bleeding and mostly elevated after the period of bleedings, 65.4 per cent/hour on an average. During the repeated bleedings the production of red cells increases to many times the resting value. In Fig. 1 the MRR is plotted against daily mean production of erythrocytes. As may be seen there is a rough correlation between the rate of methemoglobin reduction and that of erythropoiesis as long as the rate of red cell formation does not exceed 6 per cent of the actual erythrocyte count. With higher rates there is no further increase in the MRR, sometimes even a decrease to pre-bleeding level. The coefficient of correlation is highly significant $r = +0.43$, $P < 0.0027$, but the correlation is fairly loose. If the percentage of reticulocytes is similarly compared the correlation with the MRR is even lower r being $+0.32$ which corresponds to the probability level $0.02 < P < 0.05$. Very striking is again the slow reduction rate when the percentage of reticulocytes is very high, $>$ 13 per cent.

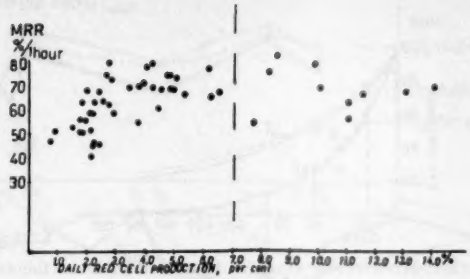


Fig. 1. Rate of methemoglobin reduction as function of red cell production.
Ordinate: MRR per cent/1 hour.
Abscissa: mean daily red cell production in per cent of actual red cell count.

Since neither the rate of erythropoiesis nor the percentage of reticulocytes can explain variations in the MRR, a tentative hypothesis was put forth, according to which the MRR in a single cell is a function of cell age and the recorded MRR can be predicted, if the age structure of the red cell population and the age decay curve of MR in a single cell are known. The construction of the last mentioned curve was based on the following considerations. The MRR was supposed to reach its highest value, 100 per cent (probably too high an estimate) on the second day after the cell has entered the circulation and the reticulocyte stage is over. It is assumed that the decrese is thereafter logarithmic and the MRR becomes zero in 160 or 180 days, *i. e.* approximately three times the mean life span of rabbit erythrocytes (Fig. 2, curves a and b). If the rate of erythropoiesis is constant and the number of cells in every age group consequently equal, the mean rate may be calculated by integrating the curves a and b and dividing with the life span of red cells. It should then give the experimentally found MRR. Curves $\bar{a}$ and $\bar{b}$ show the calculated averages and give the values 52.5 and 56 per cent at a life span of 52 days, *i. e.* reasonably correct values. The main question is now how to characterize the age structure of the red cell population in the actual blood sample. Two procedures were tried. The first method, surely oversimplified, was to calculate the mean cell age by substituting red cell destruction rate with red cell production as described in the methods and take the MRR corresponding to this "mean cell age" from curve $\bar{a}$ in Fig. 2. The result appears

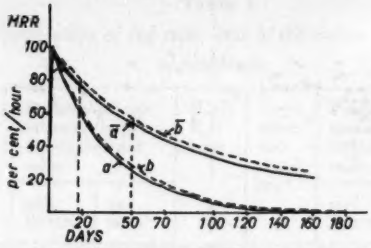


Fig. 2. Hypothetical curves indicating the decrease in MRR as a function of cell age.

Curve a: MRR decreases in a single cell logarithmically from 100 per cent on second day to 0 in 160 days.

b: the same except that 0 is reached after 180 days.

ā: mean MRR calculated by integration from curve a.

b̄: mean MRR calculated from curve b.

in Fig. 3 (dotted line) which gives the calculated MRR as a function of time during bleeding experiments with 6 rabbits. A comparison with the actually found MRR (heavy line) indicates a certain parallelism, but there are irregular deviations. They are most likely due partly to inaccuracies in the determinations of the rate of erythrocyte production, partly to the fundamental fault mentioned previously: the altered rate of erythropoiesis has been of short duration and the "mean cell age" based on it is therefore misleading. The correlation coefficient between calculated and experimental values is, nevertheless, highly significant: $r = +0.62$, $t = 5.15$, $P < 0.001$.

The second method is as follows: In our experiments a MRR of 80—85 per cent/hour was the highest obtained. 82 per cent/hour corresponds in curve b̄, Fig. 2, a cell life of 18 days. It was now calculated on basis of the production data how big a percentage of the red cells were younger than 18 days (= y) and their contribution to the mean MRR calculated by multiplying y with 82 per cent/hour. Since the mean MRR of cells aged 18—50 days is 41 per cent/hour (obtained by integration from curve b) the MRR would be $82y + (100 - y)41/100$. In Fig. 3 the variations of MRR during bleeding experiments are compared with the predicted values calculated according to this formula (continuous line, crosses). In spite of irregular discrepancies the general course of the calculated and the experimental values is the same. The correlation coefficient of calculated and experimentally found

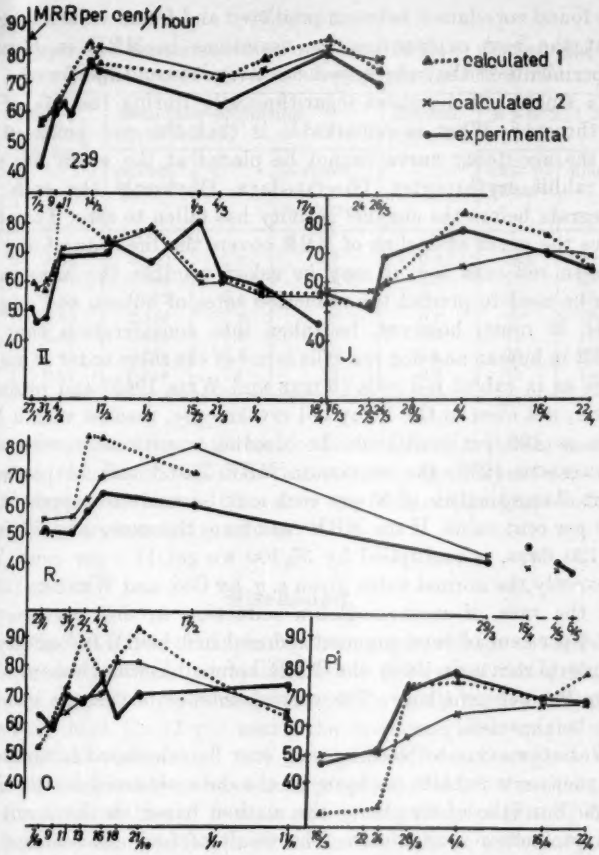


Fig. 3. Calculated and found MRR in bleeding experiments with 5 rabbits.

Heavy solid line: experimentally found rates.
Dotted line and triangles: calculated values based on "mean cell age" and curve a.
Solid line and crosses: calculated values based on percentage of red cells younger than 18 days and curve b.

values is $r = +0.55$, $t = 6.26$ which corresponds to a probability of occurrence by chance less than 0.001.

If the inaccuracy of the calculations of the rate of production of red cells is taken into consideration and, of course, the fact that the determinations of MRR are not devoid of error either

the found correlation between predicted and found values suggests that the best explanation for variations in MRR in bleeding experiments is that the enzyme activity responsible for the MR in a single cell decreases logarithmically during the life of the erythrocyte. What is remarkable is that the end point (0 %) of the age decay curve cannot be placed at the mean life span of rabbit erythrocytes, 50—60 days. Obviously the cells disintegrate before the enzyme activity has fallen to zero. Therefore, since the curve of decline of MRR covers the life span of dog and human red cells too, it may be asked whether the same curve can be used to predict the reduction rates of human and dog red cells. It must, however, be taken into consideration that the MRR in human and dog red cells is not of the same order of magnitude as in rabbit red cells (KIESE and WEIS 1943) and probably never, not even in the 1 day old erythrocyte, reaches such a high rate as 100 per cent/hour. In bleeding experiments with dogs (JALAVISTO 1959) the maximum MRR found was 31 per cent/hour. Accordingly *e. g.* 35 per cent may be made to represent the 100 per cent value. If the MRR read from the curve at a life span of 120 days, is multiplied by 35/100 we get 11.4 per cent. This is exactly the normal value given *e. g.* by COX and WENDEL (1942) for the rate of methemoglobin reduction in dog erythrocytes (11.3 per cent of total pigment reduced in 1 hour). In our experiments (JALAVISTO 1959) the MRR before bleeding was in three dogs 9.5 per cent/hour. The correspondence is thus as good as can be expected.

Variations due to bleeding may now be calculated in the same manner as in rabbits on basis of the data obtained in the three dogs. For sake of simplicity the method based on the mean red cell production was chosen. The result of such calculations are shown in Table II. In the table are taken only values based on more than a week's time interval between E″ and E′. The correspondence between calculated and experimental values is quite good if curve ā in Fig. 2 is used, curve b̄ gives somewhat too high values.

Human erythrocytes reduce likewise with approximately the same rate, the mean of five determinations so far made was 12.3 per cent/1 hour, which can be predicted since the life span of human red cells is approximately the same as that of dog cells. The age decay curve seems therefore to have general validity. It is, furthermore, not restricted to the MRR. The decrease of

Table II.

Comparison between calculated and found MRR of dog red cells, percentage of reticulocytes, and daily production of red cells.

Dog	E″ million E/mm³	Mean daily production of red cells			Reticulocytes %	MRR %/1 hour	
		million E/mm³	%	Number of days concerned		Calculated	found
F	6.25	0.102	1.6	7	0.3	16.5	15.5
»	7.07	0.205	2.9	9	2.2	22	23
M	6.10	0.153	2.5	14	0.3	20	19.5
»	6.80	0.150	2.2	8	0.9	19	18
V	6.02	0.054	0.9	78	0.8	11	17.5
»	6.61	0.206	3.1	10	0.4	23	20
»	6.44	0.089	1.4	12	0.6	15	15

cholinesterase activity in human red cells follows a similar course (ALLISON and BURN 1955). In this case too, the enzyme activity at 120 days is not zero. Obviously the cells disintegrate before the cholinesterase activity ceases.

Discussion.

That the MRR is in principle dependent upon cell age can hardly be doubted. However, it is quite obvious that when the erythrocytosis is very strongly stimulated and the percentage of reticulocytes very high (> 13 per cent), the methemoglobin might not be reduced at such a high rate as predicted from the age decay curve. Two explanations have been proposed. First it was thought that the accumulation of pyruvic acid in reticulocytes (PIHA 1958) would inhibit the reduction of methemoglobin in reticulocytes. However, since the reticulocyte stage is a very short episode in the life history of the red cell, it cannot explain the fact although the elevated concentration of pyruvic acid may last longer than the reticulum. The concentration of pyruvic acid is furthermore rather low and can not in any case entirely suppress the increase in MRR (JALAVISTO *et al.* 1959). A second and more probable explanation would be that during a very intense erythropoiesis defective cells with abnormally low enzyme activity are formed. Certain indications for the production of defective cells may be mentioned. SENO *et al.* (1953) have observed that during intense reticulocytosis part of the reticulocytes are "de-

generated" cells which do not ripen at all in *in vitro* incubation experiments. PIHA has performed measurements of plasma oxidation reduction potential in bleeding anemia of rabbits, and considers the plasma potential as an indicator of the overall metabolic activity of the red cells. The correlation between plasma potential and reticulocytosis is evident as long as the percentage of reticulocytes does not exceed 12 per cent. With higher percentages the oxidation reduction potential does not further increase or may even decrease. This is interpreted as due to the existence of inactive or functionally defective reticulated cells. It is quite remarkable that the correlation between reticulocytes and plasma potential breaks down approximately at the same percentage of reticulocytes as that between reticulocytes and MRR. LINMAN and LONG (1958) have found that during bleeding anemia a humoral factor which stimulates erythropoiesis, gives rise to quite shortlived red cells. Likewise the form of the age decay curve of the specific radioactivity of ^{14}C-labeled red cell globin differs clearly in normal and anemic (bled) rabbits suggesting the existence of a population of shortlived red cells in the bled rabbits (PIHA 1956).

The rôle of the methemoglobin reduction in red cells is usually interpreted as preventing the cell of decay which otherwise would follow the autoxidation of cell hemoglobin. Whether the life span of the erythrocyte is factually dependent upon the equilibrium between the rate of methemoglobin formation and its reduction has never been experimentally tested. If the decline in methemoglobin reduction takes the same course in rabbit, dog and human erythrocytes as seem to be the case, this hypothesis could be validated by comparing the rate of methemoglobin formation and reduction under various conditions in these three species with different life span of their erythrocytes.

Summary.

1. The dependence of the rate of reduction of methemoglobin (MRR) in nitrite treated red cells of cell age has been investigated. Starting from the assumption that the decrease of MRR with cell age proceeds logarithmically from 100 per cent at the second day to zero on the 160th or 180th day the mean MRR was calculated for different cell ages from the integrated hypothetical curve. The mean daily production of red cells in rabbits and

dogs during normal conditions and when the erythropoiesis was stimulated through repeated bleedings was calculated with help of a formula proposed by PIHA. The formula, however, gives only approximate values.

2. The age characteristics of the red cell population were defined: 1) by the "mean cell age" = 100/daily production of red cells (per cent) and 2) by estimating the percentage of younger than 18 days old cells on basis of the daily red cell production data. The mean MRR could then be predicted with help of the hypothetical curve. When the many inaccuracies involved in the estimates and primary data are taken into consideration the agreement of the predicted and found values is good, the correlation coefficients being: $r_1 = +0.62$, $t = 5.15$ $P < 0.001$ $r_2 = +0.55$ $t = 6.26$ $P < 0.001$.

3. The same logarithmically decreasing age decay curve could be used for calculation of the MRR both in rabbits with a red cell life span of 50 days and in dog and human erythrocytes with an approximate life span of 120 days if the maximum reduction rate is assumed to be 100 per cent/1 hour in rabbit and 35 per cent/1 hour in dog and human erythrocytes.

Aided by a grant from Valtion luonnontieteellinen toimikunta (State Commission of Science).

References.

ALLISON, A. C. and G. P. BURN, Enzyme activity as a function of age in the human erythrocyte. *Brit. J. Haemat.* 1955. *1.* 291—303.

COX, W. W. and W. B. WENDEL, The normal rate of methaemoglobin reduction in dogs. *J. biol. Chem.* 1942. *143.* 331.

DORNHORST, A. C., Analytical review: the interpretation of red cell survival curves. *Blood* 1951. *6.* 1284.

HUNTER, F. T., Quantitation of Mixtures of Hemoglobin Derivatives by Photoelectric Spectrophotometry. *Springfield,* Ill. 1951. pp. 226.

JALAVISTO, E., Bleeding anaemia and methaemoglobin reduction in dog erythrocytes. *Acta physiol. scand.* 1959. *46.* 252—256.

JALAVISTO, E., A. PITKÄNEN, A. SELONEN and L. SOLANTERÄ, The rate of methaemoglobin reduction in red cells of moderately bled rabbits. *Acta physiol. scand.* 1959. *46.* 257—272.

KIESE, M. and B. WEIS, Die Reduction des Hämiglobins in den Erythrocyten verschiedener Tiere. *Arch. exp. Path. Pharmak.* 1943. *202.* 494—501.

KÜNZER, W. and O. SCHNEIDER, Zur Aktivität der reduzierenden Fermentsysteme in den Erythrocyten junger Säuglinge. *Acta haemat.* (*Basel*) 1953. *9.* 346—353.

LINMAN, J. W. and M. J. LONG, Erythrocyte osmotic fragility of rats receiving the thermostable plasma erythropoietic factor. *Blood* 1958. *13.* 226—238.

PIHA, S., Studies on blood regeneration during progressive reticulocytosis due to repeated bleedings. *Ann. Acad. Sci. Fenn.* 1956. Ser A. II. 80 pp. 99.

PIHA, R. S., Behaviour of pyruvic acid in plasma and red cells in rabbits bled repeatedly. *Acta physiol. scand.* 1958. *44.* Suppl. 151. pp. 43.

SENO, S., K. KAWAI and K. NISHIKAWA, Maturation of reticulocytes and related phenomena. III. Maturation of reticulocytes in vitro; observation on anemic rabbits. *Mie Med. J.* 1953. *4.* Suppl. 1. 19—34.

Acta physiol. scand. 1959. *46*. 284—290.

From the Department of Physiological Chemistry, University of Lund, Lund, Sweden.

Bile Acids in Rat Portal Blood.

Bile Acids and Steroids 77.

By

THOMAS OLIVECRONA and JAN SJÖVALL.[1]

Received 2 February 1959.

Abstract.

OLIVECRONA, T. and J. SJÖVALL. Bile acids in rat portal blood. *Acta physiol. scand.* 1959. *46*. 284—290. — Tritium-labelled cholic acid was given to four rats and radioactive bile acids in the portal blood were analyzed with reversed phase partition chromatography after two days. One tenth to two tenths per cent of the radioactivity mixed with the bile acid pool was present in one ml of portal blood. About 15 per cent of the radioactive portal bile acids were unconjugated, approximately 50 per cent of which was deoxycholic acid as judged by its chromatographic behaviour. These figures have been used for rough calculations of the extent of enterohepatic circulation and cecal absorption of cholic acid and its metabolites, and for the estimation of the daily conversion of cholic acid into deoxycholic acid by intestinal microorganisms.

Previous studies of the portal bile acids have been limited to the determination of cholic acid and it has been shown that less than one per cent of the bile acids absorbed from the intestine are transported in the lymph (JOSEPHSON and RYDIN 1936, SJÖVALL

[1] Present address: Statens Rättskemiska Laboratorium, Stockholm 60.

and ÅKESSON 1955). Recently, it has been observed that some bile acids normally found in the bile are formed through the action of intestinal microorganisms during the enterohepatic circulation of the bile acids, primarly formed from cholesterol in the liver (LINDSTEDT and SJÖVALL 1957, LINDSTEDT 1957, NORMAN and SJÖVALL 1958). In the rat, such acids are formed mainly in the cecum from which they can be absorbed (NORMAN and SJÖVALL 1958). In order to estimate the extent of absorption of these bacterial metabolites in the intact rat, we have made chromatographic analyses of the bile acids in rat portal blood two days after the intraperitoneal administration of tritium labelled cholic acid. The results are reported in this paper.

Experimental.

White rats of the institute stock weighing about 200 g were used. They were injected intraperitoneally with approximately 1 mg of tritium labelled cholic acid (BERGSTRÖM and LINDSTEDT 1957) giving about 100×10^6 cpm. when counted in a flow-counter (Frieseke-Hoepfner FH 51). This acid was kindly supplied by Dr. Lindstedt.

The rats were given white bread and water, and two days after the administration of the labelled acid portal blood was collected by puncture of the portal vein with a plastic cannula as near to the liver as possible. The animal was kept in a light ether anesthesia. Four to 5 ml of blood were obtained and allowed to drain directly into a test tube containing heparin.

The heparinized blood was then added drop by drop to 100 ml of 96 % ethanol and heated on the water bath for about half an hour. After cooling, the extract was filtered and the precipitate washed with ethanol. The ethanol was evaporated *in vacuo*. This procedure produced a complete recovery of labelled deoxycholic, glycocholic and taurodeoxycholic acids added to blood.

The intestine was taken out after collection of the portal blood. Small and large intestines were cut into small pieces and extracted separately by refluxing three times with 80 % ethanol. Similarly, the feces were extracted with 80 % ethanol.

The radioactivity of the extracts was determined by counting in an infinitely thin layer (10 μg per cm^2) on aluminium planchets; a flow-counter (Frieseke-Hoepfner FH 51) was used.

The ethanol extract of the portal blood was extracted with butanol from an acidified water solution (NORMAN 1954). It was then subjected to a reversed phase chromatography with 55 % (v/v) methanol/water as moving phase and 10 % (v/v) heptane/chloroform as stationary phase, supported on 4.5 g hydrophobic kiselguhr (phase system F 1). In this way free deoxycholic acid (appearing at 50 ml effluent) and more hydrophobic bile acids are separated from free cholic acid and more

Table I.

Rat	Cpm. recovered in portal blood, liver and intestines	Activity per ml portal blood per cent of total	Per cent of activity in portal blood appearing in the chromatograms at the places corresponding to:			
			Conjugated bile acids	Free bile acids		More polar than cholic
				Deoxycholic	Cholic	
P 1 ..	—	—	94	4	1	1
P 2 ..	54.5×10^6	0.20	86	5	1	8
P 3 ..	61.5×10^6	0.19	90	5	1	4
P 4 ..	45.2×10^6	0.13	73	16	3	8

polar bile acids including conjugated acids which appear with the front. The front band from this chromatography was then run on a column where 48 % (v/v) methanol/water was used as moving phase and 50 % (v/v) isooctanol/chloroform as stationary phase (phase system C 2). In this chromatography the taurine conjugated bile acids came with the front, free cholic acid at about 120 ml effluent and free acids more polar than cholic acid between these two bands.

The radioactivity of all the fractions collected from the column was determined and the proportions between the different bile acids were calculated from the total radioactivity in the different bands.

Results.

The distribution of radioactivity in the intestinal tract was the same as that which was reported earlier (NORMAN and SJÖVALL 1958), *i. e.* about 70 % in the small intestine and 30 % in the large intestine. The activity in the liver was not determined but could not have amounted to more than a few per cent of the total activity (NORMAN and SJÖVALL 1958). The portal blood contained 0.90, 0.80 and 0.53 % of the total radioactivity in three rats studied, giving 0.20, 0.19 and 0.13 % respectively per ml blood (Table I). The radioactive material from the blood was separated with reversed phase chromatography. Radioactive bands were found at the places corresponding to conjugated bile acids as present in the small intestine and at the places corresponding to the main cholic acid metabolites known to be formed in the cecum (NORMAN and SJÖVALL 1958). Fig. 1 shows the chromatograms obtained with the material from rat P 2. The chromatography with phase system F 1 gave a band of activity with the front and another band at the place of free deoxycholic acid (Fig. 1 left curve).

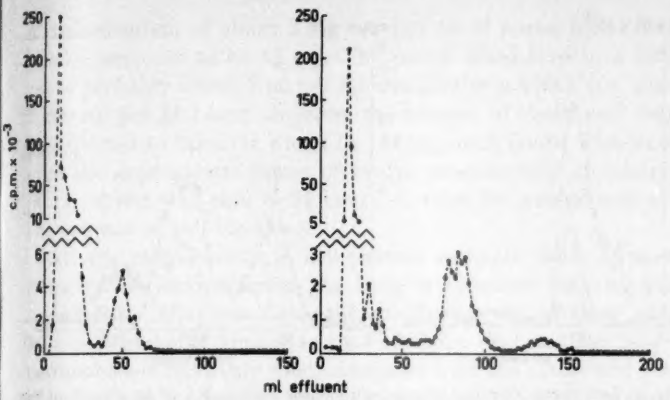


Fig. 1. Chromatograms of the extract of portal blood from rat P 2.

Left curve: Chromatography with phase system F 1.
Right curve: Rechromatography with phase system C 2 of the front band of the chromatography with phase system F 1.

Rechromatography with phase system C 2 of the front band (Fig. 1, right curve) showed active peaks at the places corresponding to conjugated bile acids (front band), unconjugated 7-ketodeoxycholic and/or 3 α, 7 β, 12 α-trihydroxycholanic acids (more polar than cholic acid) and free cholic acid. Essentially the same results were obtained with the other rats and the relative proportions of radioactivity in the various bands are summarized in Table I. Since it is known that the above mentioned free bile acids, with the possible exception of 3 α, 7 β, 12 α-trihydroxycholanic acid, are formed from conjugated cholic acid in the cecum and can be found reconjugated in the bile (NORMAN and SJÖVALL 1958), only rechromatographies with the appropriate unlabelled reference compounds were made for further identification. The "deoxycholic acid band" appeared together with deoxycholic acid and the "cholic acid band" together with cholic acid, preceded by acids more polar than cholic acid. Fig. 2 shows chromatograms of aliquots of the combined free bile acid bands from the different rats. Except for the minor peak of radioactivity after cholic acid (Fig. 2, right curve) that cannot be explained, the peaks agree well with the peaks obtained using extracts of cecal contents (NORMAN and SJÖVALL 1958).

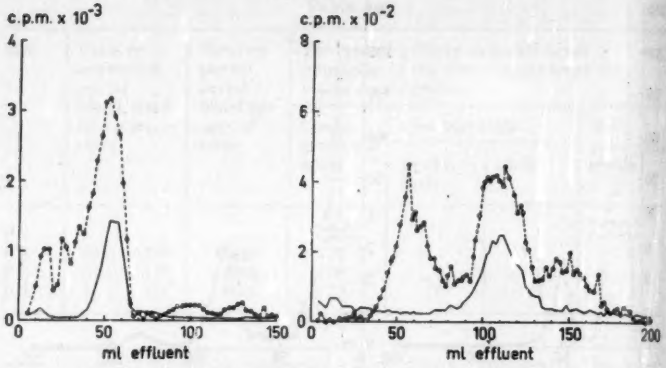


Fig. 2. Left curve: Chromatography with phase system F 1 of an aliquot of the combined "deoxycholic acid bands" together with unlabelled deoxycholic acid.

Right curve: Chromatography with phase system C 2 of an aliquot of the combined "cholic acid and more polar than cholic acid bands" together with unlabelled cholic acid.

Solid lines: Titration.

Broken lines: Radioactivity.

There were large variations between the rats, but approximately 15 % of the radioactivity was found in the bands corresponding to free bile acids and half of this amount in the deoxycholic acid band (Table I). Chromatographic analysis of the conjugated bile acids, after hydrolysis, yielded chromatograms similar to those obtained with hydrolyzed small intestinal contents.

Discussion.

Although the number of animals used was small, certain approximate estimations of the enterohepatic circulation of cholic acid may be made from the data obtained. The cholic acid pool in a rat of the size used in this work (200 g) has been found to be about 12 mg (ERIKSSON 1957). This pool is present mainly in the intestine, and when labelled cholic acid is injected intraperitoneally, it is rapidly mixed with the pool (NORMAN and SJÖVALL 1958). If the radioactivity in the intestine is taken to represent the bile acid pool, a mean of 0.17 % of the pool is present in one ml of portal blood in the rats examined. This would mean

a concentration of about 2 mg per 100 ml of portal blood if the pool is assumed to be 12 mg. The portal blood flow in a 200 g rat is probably about 5 ml per minute (SPECTOR 1956), *i. e.* about 7,200 ml per 24 hours, therefore the amount of cholic acid being transported to the liver would be 144 mg in 24 hours. This would give the approximate figure of twelve enterohepatic circulations of the cholic acid pool in 24 hours. It must be remembered that the rat has no gall-bladder.

All the radioactivity is not present as cholic acid, however. Some of the acid injected has been transformed into bacterial metabolites. After two days, the specific activity of these metabolites is probably similar to that of cholic acid and the pool of metabolites is relatively small compared with the cholic acid pool. Therefore the presence of metabolites will not affect the approximate estimations to any great extent.

BERGSTRÖM and DANIELSSON (1958) have found that the formation of bile acids in the liver is regulated by the amount of bile acids supplied to the liver via the portal blood. About 5 mg of bile acids per hour had to be supplied to the liver of bile fistula rats to keep the bile acid synthesis on the level found in intact animals. This would indicate a daily transport of about 120 mg of bile acids in the portal blood, a figure that agrees well with our calculations.

Approximately 15 per cent of the bile acids in the portal blood are unconjugated. Most likely these free bile acids have been absorbed from the cecum, where almost all bile acids are unconjugated in contrast to those present in the small intestine (NORMAN and SJÖVALL 1958). Thus, about 20 mg of the bile acids transported to the liver in 24 hours have probably come from the cecum, which would mean that the cholic acid pool (and cholic acid metabolites) passes the cecum about twice in 24 hours during the enterohepatic circulation.

Deoxycholic acid formed from cholic acid by intestinal microorganisms (NORMAN and SJÖVALL, 1958) comprises about half the amount of the free bile acids. Approximately 10 mg of deoxycholic acid therefore is transported to the liver in 24 hours and the major part is again transformed into cholic acid in the liver (BERGSTRÖM, ROTTENBERG and SJÖVALL, 1953). Under such circumstances, about 80 per cent of the cholic acid pool would be converted to deoxycholic acid and reconverted to cholic acid during 24 hours of enterohepatic circulation. This figure agrees

with the investigation by LINDSTEDT and SAMUELSSON (1958), who arrived at a similar figure measuring the decrease of the tritium/C^{14} ratio after injection of cholic acid-7 β T-24-C^{14}, which lost the tritium when converted to deoxycholic acid and back to cholic acid.

Although the figures discussed above must be regarded as very proximal, they will at least give a rough quantitative picture of the enterohepatic circulation of cholic acid, the importance of absorption from the cecum, and the interaction between the intestinal flora and the liver in the metabolism of cholic acid to deoxycholic acid and back to cholic acid.

The technical assistance of Mrs. ANN-MARI ANDERSSON is gratefully acknowledged.

This work is part of investigations supported by the Swedish Medical Research Council and by a research grant H 2842 from the Heart Institute, Public Health Service, U. S. A.

References.

BERGSTRÖM, S. and S. LINDSTEDT, A note on the tritiation of organic compounds in tritium gas. *Acta chem. scand.* 1957. *11*. 1275.

BERGSTRÖM, S. and H. DANIELSSON, On the regulation of bile acid formation in the rat liver. *Acta physiol. scand.* 1958. *43*. 1—7.

BERGSTRÖM, S., M. ROTTENBERG and J. SJÖVALL, Über den Stoffwechsel der Cholsäure und Desoxycholsäure in der Ratte. *Hoppe-Seyl. Z. physiol. Chem.* 1953. *295*. 278—285.

ERIKSSON, S., Biliary excretion of bile acids and cholesterol in bile fistula rats. *Proc. Soc. exp. Biol. (N. Y.)* 1957. *94*. 578—582.

JOSEPHSON, B. and A. RYDIN, The resorption of the bile acids from the intestines. *Biochem. J.* 1936. *30*. 2224—2228.

LINDSTEDT, S., The formation of deoxycholic acid from cholic acid in man. *Arkiv Kemi*. 1957. *11*. 145—150.

LINDSTEDT, S. and B. SAMUELSSON, On the interconversion of cholic and deoxycholic acid in the rat. *J. biol. Chem.* 1959. In press.

LINDSTEDT, S. and J. SJÖVALL, On the formation of deoxycholic acid from cholic acid in the rabbit. *Acta chem. scand.* 1957. *11*. 421—426.

NORMAN, A., On the conjugation of bile acids in the rat. *Acta physiol. scand.* 1954. *32*. 1—10.

NORMAN, A. and J. SJÖVALL, On the transformation and enterohepatic circulation of cholic acid in the rat. *J. biol. Chem.* 1958. *233*. 872—885.

SJÖVALL, J. and I. ÅKESSON, Intestinal absorption of taurocholic acid in the rat. *Acta physiol. scand.* 1955. *34*. 273—278.

SPECTOR, W. S., Handbook of biological data. Phil. & Lond. 1956.